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*ACCELERATED DNA SYNTHESIS IN ONION ROOT MERISTEM DURING X-IRRADIATION**

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Communicated by Daniel Mazia, November 22, 1960

It has been demonstrated recently that deoxyribonucleic acid (DNA) contents of X-irradiated mammalian cells continue to rise to the premitotic level in the absence of mitosis.[1–3] This is in contrast to the widespread belief that interference with DNA biosynthesis is one of the primary actions of radiation.[4–7] The present report deals with the effects of X-rays on cell proliferation, DNA synthesis, and the nucleohistone complex in onion root tips. As it had been found to do in animal materials (*loc. cit.*), DNA duplication continues to occur in these plant cells after irradiation; moreover, it will be demonstrated that DNA synthesis is actually stimulated during the process of irradiation.

Material and Methods.—Seed onions with roots $1^1/_2$ to 2 inches long were irradiated with 200 r, 800 r, 2000 r, and 3,200 r at a dose rate of 50 r per minute (at 250 kV and 15 mA, using 0.25 mm Cu and 1.0 mm Al filters). During irradiation, roots were completely submerged in ordinary tap water or in tap water containing tritiated thymidine (0.4 μc/ml; spec. act.: 1.9 C/mM). Immediately after irradiation, roots were allowed to grow in tap water with or without added tritiated thymidine. Parallel controls with nonirradiated roots were also run. Roots were fixed for six hours in Baker's neutral formalin or in acetic acid alcohol fixatives at frequent intervals starting from zero and continuing up to 48 hours post-radiation. Formalin-fixed tissues were washed in running water for about six hours. All samples were stored in 70 per cent ethanol.

Twelve-micron paraffin sections of formalin-fixed tissues were used for microspectrophotometric determinations of DNA and histone in the same individual nuclei. Sections from all tissues were hydrolyzed together for eight minutes in 1 *N* trichloroacetic acid (TCA) at 60°C and stained with TCA-Feulgen.[8] DNA contents of randomly selected 1,230 uncut interphase, 20 late telophase, and 20 early prophase nuclei were measured at 6,000 Å. After the completion of DNA measurements, the sections were stained with alkaline fast-green[9] for photometric measurements of histone (also at 6,000 Å) in some of the same individual nuclei whose DNA contents had been determined and which had been mapped prior to measuring. Mitotic counts were also made on these sections. Acetic alcohol-fixed squash preparations of about 1 mm tips (excluding root caps) were used exclusively for making autoradiographs by the stripping film technique and for further mitotic counts. The frequency of radioactive nuclei was determined, and the number of silver grains over many randomly selected interphase nuclei were counted. In order to correlate the incorporation of tritiated thymidine with the synthesis of DNA, photometric Feulgen-DNA determinations and grain counts on the same individual interphase nuclei (711) were also made on some squash preparations.

Results.—(a) *Nuclear DNA and histone contents in control and irradiated cells:* The photometric DNA measurements of late telophase and early prophase nuclei

from nonirradiated control roots yield two distinct DNA classes (2C and 4C) in a ratio of 1 : 2. Three hundred and thirty interphase nuclei from 33 control roots show, in addition to these two classes, many intermediate DNA values representing nuclei in the process of DNA synthesis prior to their entrance into mitosis. The

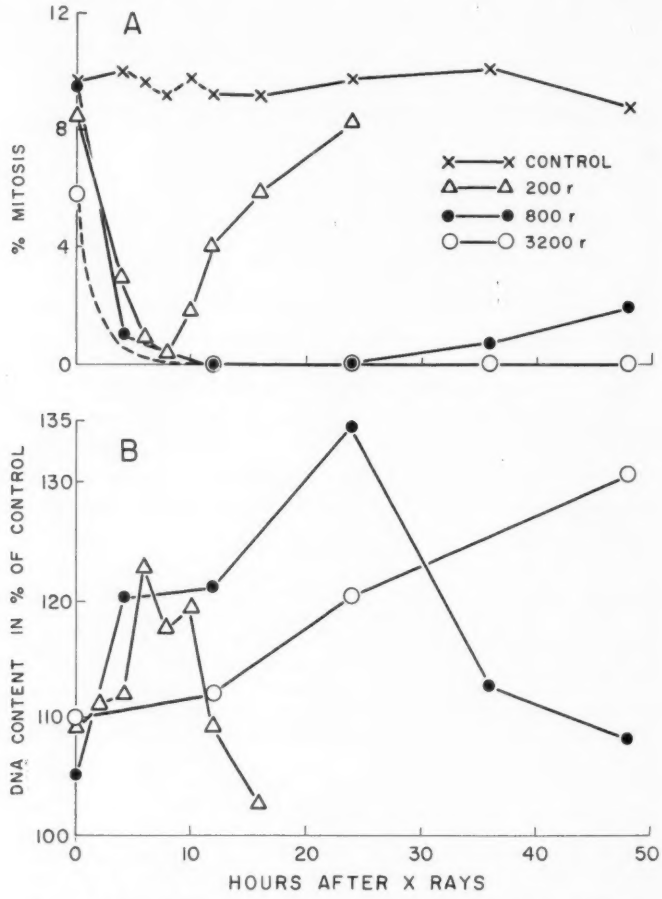

FIG. 1.—Changes in mitotic rates (A) and in average Feulgen-DNA contents (B) as a function of time. A: Number of roots per point = 3 (except 2 at 10 hours after 200 r and at 24 and 48 hours after 800 r) with a mean number of 387 cells scored per root. B: Control value (i.e., base line) obtained from 330 nuclei in 33 roots; number of roots per point is same as in (A); for each point, from 30 to 60 nuclei were measured.

frequency of 2C interphase nuclei (50%) in these roots is higher than that of 4C (37%).

The frequencies of 4C nuclei in tissues collected immediately after irradiation (i.e., at time zero) with 200 r, 800 r, and 3,200 r are 57, 47, and 48 per cent, respectively. This change in the distribution of DNA values toward the higher class raised sig-

nificantly the average DNA contents of the populations of irradiated nuclei up to about 10 per cent over the control value (see Fig. 1). The initial increase in DNA values of irradiated roots does not seem to be due to an immediate block of mitosis, because at time zero the mitotic rates after 200 r(8.4 ± 1.01) and 800 r(9.4 ± 0.49) are approximately the same as in simultaneous controls (9.6 ± 1.22). It is, however, lower after irradiation with 3,200 r(5.9 ± 1.06), a dose which required a sufficently long administering time (64 minutes) for the mitotic block to take effect.

The changes in mitotic indices and in average DNA values of nuclear populations with time after irradiation are graphically represented in Figure 1. The increase

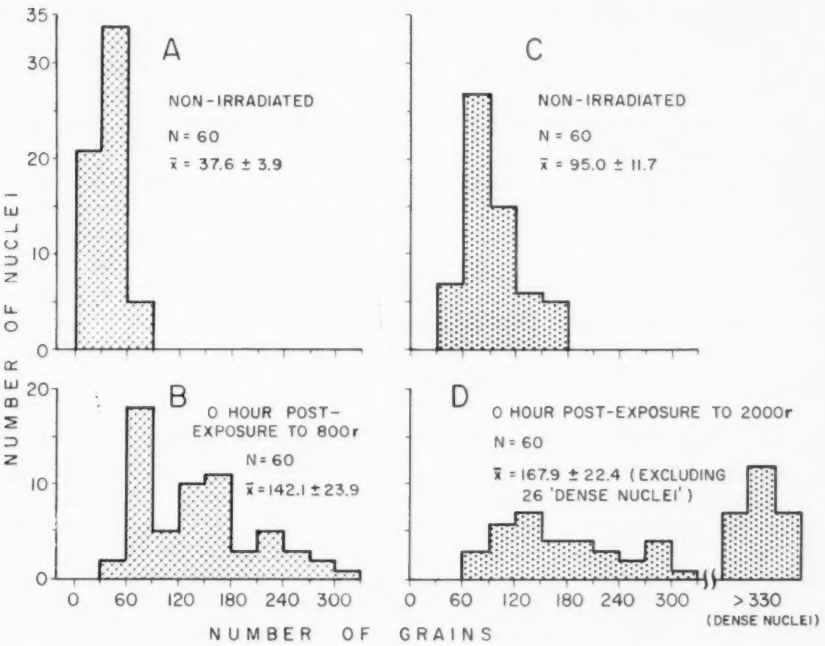

FIG. 2.—Distribution of silver grains over irradiated and non-irradiated nuclei. During irradiation (at 50 r/min) roots were submerged in tap water with 0.4 μc/ml of thymidine-H^3. Exposure to thymidine-H^3: A and B for 16 minutes; C and D for 40 minutes. All autoradiographs exposed for six weeks. N = number of nuclei (20/root); $\bar{x}$ = mean grain number/nucleus.

and eventual decrease (toward control levels) of DNA values in roots irradiated with 200 r and 800 r correspond directly to the inhibition and recovery of the mitotic rates. Roots exposed to 3,200 r, in which mitosis does not recover, also exhibit permanently increased DNA values; the slower rate of DNA increase in this last group is possibly due to the death of many 2C cells in this population.

Fast-green measurements of histone in 20 mitotic and 20 interphase nuclei from control tissues, whose DNA contents had been determined previously, show a DNA-histone ratio of 1.03 ± 0.01. Forty nuclei from roots collected 6 and 16 hours after irradiation with 200 r and 40 from roots sampled 12 and 48 hours after exposure to 3,200 r give DNA-histone ratios of 1.03 ± 0.01 and 1.01 ± 0.01, respectively.

These ratios do not differ from that of control roots. Hence, the nucleohistone complex of the irradiated nuclei exhibits normal cytochemical characteristics.

(b) *Incorporation of tritiated thymidine:* It has been mentioned above that the average DNA values increase during irradiation. Since the mitotic rates in these tissues immediately after irradiation are about the same as those of the unirradiated controls, it suggests that DNA synthesis is stimulated during irradiation. Additional experiments have, therefore, been performed to determine whether there is an increased incorporation of tritiated thymidine into DNA during irradiation. It can be seen in Figure 2 that roots which are irradiated in the presence of tritiated thymidine for 16 and 40 minutes (total doses 800 r and 2000 r, respectively) and collected immediately after irradiation show a very high increase, compared to controls, in the incorporation of tritiated thymidine. In the former, the number of silver grains over an average nucleus is up to four times more than in the control (Fig. 2 A and B); in the latter, it is about two times more, even after excluding 16 (out of 60) nuclei which produced such high densities of silver grains that exact counts could not be made (Fig. 2 C and D).

Photometric DNA measurements and silver grain counts on the same nuclei reveal that the increased incorporation of tritiated thymidine during irradiation does not just represent increased thymidine utilization but corresponds to increased synthesis of Feulgen-stainable DNA. The estimation of the ratio R of the average amounts of DNA synthesized in time t in X-rayed samples to the average amounts in control samples can be made from the following equation:

$$R = 1 + \frac{\bar{X}_i - \bar{X}_c}{\bar{X}_t},$$

where $\bar{X}_i$ and $\bar{X}_c$ are the mean Feulgen-DNA values of irradiated and control samples respectively, and $\bar{X}_t$ is the fraction of DNA synthesized by an average control nucleus in time t. To obtain the value of $\bar{X}_t$, it is necessary to know the class value of 2C nuclei and the time necessary for such a nucleus to complete its DNA duplication. The 2C class value is obtained by measuring DNA of telophase nuclei, and the time required for an average onion root tip nucleus to undergo DNA doubling appears to be about six hours, like that of *Vicia faba* root tip nuclei [5] in

TABLE 1

A CORRELATION OF GRAIN COUNTS WITH DNA SYNTHESIS IN THE SAME INDIVIDUAL NUCLEI

	Treatment (50 r per min)	No. of roots	No. of nuclei	Mean grain no. per nucleus	Mean DNA content per nucleus	Grain ratio (X-rayed to control)	Ratio* of newly synthesized DNA (X-rayed to control)
I	Control	1	42	20.4	35.67	1.7	2.0
	2,000 r	1	39	35.2	38.12		
II	Control	3	86	11.5	48.18	5.2	4.5
	800 r	3	134	59.9	51.68		

* Calculated from $R = 1 + \frac{\bar{X}_i - \bar{X}_c}{\bar{X}_t}$, the ratio of average amounts of DNA synthesized in time t in X-rayed samples to average amounts in control samples (see text). Squash preparations were stained with Feulgen; in experiment (I), grain counts preceded DNA measurements; in (II), DNA measurements preceded application of stripping film. Autoradiographs were exposed for 1 and 4 weeks, respectively. DNA values in column 5 are given in arbitrary units of Feulgen-dye content, which, for technical reasons, differed in the 2 experiments.

which the durations of the total mitotic cycle and of the post-synthetic interphase period are similar to onion. Table 1 reports the results of two experiments in which grain ratios and ratios of newly synthesized DNA of irradiated nuclei to those of control nuclei were compared by making both types of measurements on the same nuclei. In each of the two experiments, these ratios agree fairly well with one another. The six-hour total synthesis time used in this calculation is likely to be a minimal estimate; if DNA synthesis takes longer, the values of R would increase and become larger than the grain ratios. Factors other than net synthesis would only have to be invoked, however, if the grain ratios were appreciably larger than the corresponding R values.

An analysis of frequencies of radioactive nuclei in nonirradiated and irradiated tissues reveal that these frequencies are significantly increased in tissues collected immediately after exposure to X-rays (Fig. 3). When such irradiated tissues are allowed to grow in tap water containing tritiated thymidine for an additional hour, these frequencies become similar to those from the corresponding controls.

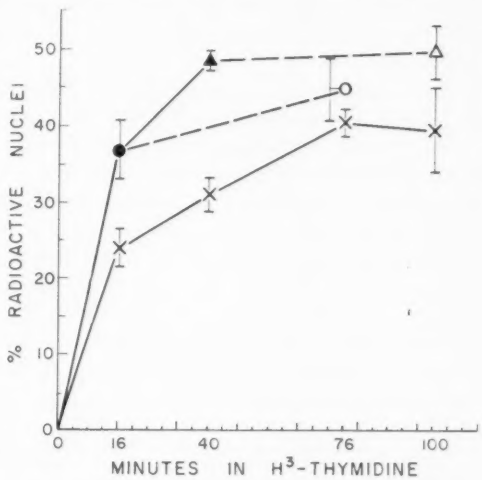

FIG. 3.—Frequency of radioactive nuclei from nonirradiated and irradiated (dose rate 50 r/min) roots as a function of time of exposure to thymidine-H^3. Each point represents 3 roots (except 2 at 1 hour after 800 r) with a mean number of 205 nuclei counted per root. ×: control samples; ●, ○: zero and one hour samples, respectively, after 800 r; ▲, △: zero and one hour samples, respectively, after 2,000 r. All autoradiographs exposed for six weeks.

Discussion.—In agreement with other studies, it is found that X-irradiation inhibits mitosis in these root tip cells. DNA synthesis, on the other hand, continues during a period of complete mitotic arrest. Howard[10] and Kelly[6] state that DNA synthesis may be relatively insensitive to radiation and that the decreased synthesis after exposure to X-rays could be a secondary consequence of mitotic arrest and cell death, since these will bring about a change in the cell population. The microspectrophotometric as well as autoradiographic data on DNA synthesis from the present study reveal not only that DNA replication does continue after irradiation, but also that the rate of synthesis is increased during irradiation. Furthermore, an additional number of 2C nuclei appears to be triggered into DNA synthesis during irradiation. It is not yet known how these effects are brought about by X-rays. That DNA synthesis should be accelerated in consequence of a treatment known to damage DNA is perhaps not too surprising in view of the finding that "damaged" DNA can be a very efficient primer of DNA synthesis in *in vitro* systems.[11]

It has been reported by many workers (see Stocken[7] for a recent review) that in cells which are supplied with labeled DNA precursors after X-irradiation a reduction

in precursor uptake into their DNA takes place. From the present results, it seems clear that such a reduction could be a consequence of the acceleration of DNA synthesis and the mitotic inhibition resulting from irradiation. These two effects in combination are bound to lead to a reduction in the frequency of 2C nuclei capable of entering DNA synthesis and consequently to reduced overall precursor uptake some time after irradiation. This could also explain the disappearance of intermediary Feulgen-DNA values from onion root tips collected several hours after X-irradiation, as reported by Swift.[12]

Rasch and Woodard[13] have recently demonstrated that the DNA-histone ratio remains constant in a variety of normal and pathological plant tissues. That this ratio in onion root tip cells remains unaltered after X-radiation has been shown in the present study. Pycnotic changes which produce a changed Feulgen/fast-green ratio in nuclei of damaged animal cells[14] do not occur in these root tips within 48 hours after irradiation.

The present results, thus, demonstrate that X-ray inhibition of mitosis in onion root tip cells is not mediated through a blockage on DNA synthesis or reflected in changes in the nucleohistone complex. It is not clear yet whether the accelerated DNA synthesis that takes place during X-ray treatments is one of the causes for this inhibition or whether it is due to an entirely different process.

Summary.—The effects of various doses of X-rays on synthesis of DNA and on the nucleohistone complex in individual onion root tip cells has been studied using both microspectrophotometric and autoradiographic methods.

Cell proliferation is inhibited by X-rays. The process of DNA synthesis, on the contrary, is stimulated during irradiation and continues to the premitotic level in a period of complete mitotic inhibition. The normal cytochemical properties of the nucleohistone complex appear unchanged even after heavy irradiation.

The authors wish to thank Drs. L. S. Kelly and S. R. Pelc for their valuable suggestions during this study.

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*THE CIS-TRANS ISOMERIZATION OF CONJUGATED POLYENES AND THE OCCURRENCE OF A HINDERED CIS-ISOMER OF RETINENE IN THE RHODOPSIN SYSTEM**

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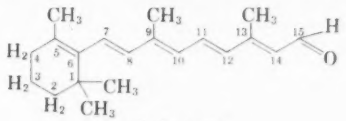
Communicated by Albert Szent-Györgyi, November 14, 1960

It is well known, since the work of Wald,[1] that the visual pigments involve the combination of a protein, opsin, with the molecule of retinene (I), the aldehyde of vitamin A. Like all polyene chains, retinene and vitamin A can exist in a number of different configurations, corresponding to the possible *cis-trans* isomerizations around the different double bonds of these molecules. In the case of (I), these possible isomerizations may occur essentially around the bonds 9–10, 11–12, and 13–14. The names given to the principal isomers are summed up in Table 1.



(I) *Retinene*

TABLE 1

Isomerization around bonds	Nomenclature	Name
9–10	9-*cis*	iso-*a*
11–12	11-*cis*	neo-*b*
13–14	13-*cis*	neo-*a*
9–10, 13–14	9, 13-di-*cis*	iso-*b*
11–12, 13–14	11, 13-di-*cis*	neo-*c*

It has been considered for many years that the existence of the 11-*cis* isomer was very improbable because the steric interference of the methyl group fixed at carbon 13 with the hydrogen at position 10 would prevent the molecule from being entirely planar,[2] thus causing a loss of resonance energy, which means a loss of stability.[3] It was consequently surprising, at least at first sight, to observe that the very isomer of retinene which is involved in the natural visual pigments has in fact the 11-*cis* configuration.[4] Moreover, it was also shown that when the all-*trans* retinene is irradiated with white light, the 11-*cis* isomer is formed about 8 times as fast as the 9-*cis* isomer.[5] However, at the same time, a comparison of the all-*trans*, 9-*cis*, 11-*cis*, 13-*cis* and 9,13-di-*cis* retinenes shows[6] that the 11-*cis* compound is the most thermolabile of these molecules (when incubated 3 hr at 70°C in 1 per cent digitonin solution). This isomer is also the most labile upon irradiation by light of $\lambda > 410$ mμ (u.v. excluded)[6] and upon iodine-catalysed irradiation.[7]

These facts tend to indicate that if the "hindered" isomer is perhaps the least stable, it is, however, the most easily formed under various conditions.[6, 8] Obviously, a careful distinction must thus be made between the stability of the isomers and the ease of their formation. It appears to us that such a distinction is of particular importance for the problem of the biochemical role of retinenes. The fact that the "hindered" isomer appears in nature in the rods and cones of the retina

is not irreconcilable with the possible instability of its isolated form, as this particular isomer may well be trapped by opsin at its very formation and appreciably stabilized by the combination with the protein.[9] It is consequently possible that it is the mere ease of formation of this isomer which is of preponderant importance for its biochemical role.

Moreover, it appears also possible that the "hindrance" of the type occurring in the 11-*cis* isomer of retinene is of a more limited importance for molecular stability than originally thought. This may be so because this hindrance does not need to cut the conjugation between the two parts of the molecule entirely. It is possible that the CH_3 group is itself pushed out of the molecular plane so as to leave the whole conjugated system coplanar to a large extent. Thus, a number of such "hindered" isomers of polyenes have been synthetized and are stable.[10] However, by calculating explicitly the loss of resonance energy of a polyene chain when the conjugation is cut into two parts by isomerization around a double bond adjacent to a methylated carbon, Pauling[3] was able to show that this loss should be greater for a long carotenoid molecule than for a short polyene chain. This led to the conclusion that "hindered" *cis*-isomers should be less probable for the long chains.

Whatever the state of affairs concerning the stability of the isomers may be, it appears obvious that it is essential to complete the study of the retinene problem with an investigation of the mechanism and rate of the isomerizations themselves. This is the aim of this paper.

The Activation Energies for Trans-Cis Isomerizations of Polyenes.—The problem consists, in fact, in the search of a quantity which could be a measure of the ease of isomerization from the all-*trans* structure of a polyene to one of its *cis*-configurations, i.e., a measure of the corresponding activation energy. Unfortunately, the calculation of the absolute activation energies for such transformations is not feasible at present. However, what we may hope to be able to evaluate are the *relative* activation energies for such transformations when those occur upon the different double bonds of the same polyene or in related polyenes. This can in fact be done by an application to this problem of the same kind of approximation that has been used extensively during recent years for the general interpretation of the chemical reactivity of conjugated organic molecules. This approximation consists in the so-called "method of localization energies," originally introduced by Wheland[11] and subsequently developed and used by a number of authors,[12, 13] which appears today as one of the most successful procedures for the interpretation of a number of aspects of chemical reactivity in conjugated molecules.

Following the general pattern of this method, we may extend its application to the problem of the isomerization reaction in the following way. As we are in practice interested only in the comparison of the rates of isomerizations around different bonds of the same compound or around corresponding bonds in homologs, we may admit that the main *variable* part of the activation energies for these isomerizations originates from the variation of the energy of the mobile electrons between the initial and the transition state. The evaluation of these variations necessitates then the choice of a representation of the transition state, at least as far as the π-electrons of the system are concerned. This can be reached by considering that in order to undergo a *trans* to *cis* isomerization around a double bond, the

system has to pass through a stage in which the two π electrons of the bond involved are uncoupled (when their electronic clouds point in directions perpendicular to each other). This stage may be taken as corresponding to the transition stage of the reaction, and consequently we may take as the relative measure of the ease of isomerization the energy needed for bringing the molecule to this particular state. Since each of the uncoupled electrons remains conjugated with the molecular fragment on its side, the representation of the transition state involves two such radical fragments. Thus, the energy that we have to spend in order to bring the molecule to this state is (as far as the π-electrons are concerned) the difference between the energy of the whole conjugated initial system and the sum of the energies of the two odd-numbered fragments. We shall call this quantity the *localization energy for a trans-cis isomerization* (L.E.I.). The general success of the method of localization energies supports the idea that it should be a good index for the determination of the relative values of activation energies for *trans-cis* isomerization reactions.

It may be remarked that this representation needs no specification about the detailed nature of the transition state, in particular, none as to whether a triplet state is or is not involved. Such a specification may be reached, if desired, in a higher approximation, simply by choosing a proper value for the parameter, β, in which the localization energies are evaluated (see below) in a way similar to what can be done for spectroscopic transitions.[13]

It may also be added that *s-cis*, *s-trans* isomerizations (isomerizations around a single bond) may be dealt with in a similar way, the calculation of the localization energy involving in this case the difference between the energy of the initial conjugated system and two subsystems which are two even-numbered fragments.

The method has been applied in the first place to some simple types of compounds. Table 2 gives the values of the localization energies for the isomerization around the different double bonds of the hydrocarbons C_nH_{n+2} from $n = 6$ (hexatriene) to $n = 16$. All the energy calculations have been carried out in the L.C.A.O. approximation of the molecular orbital method.[14] It should be mentioned here that we are perfectly aware of the drawbacks of this simple approximation in the case of polyene chains,[15] particularly of the fact that it yields bond orders which tend to equalize too rapidly as one moves towards the middle of a long chain. This difficulty could have been avoided by taking into account the variation of the resonance integral β[14, 16] as a function of the alternation of the single and double bonds. However, it can easily be seen that such a refinement would simply result in the accentuation of the differences between the values of the localization energies in the central bonds of the molecules, but would not change their relative order.

TABLE 2

Polyene (n)	Double bond 1	2	3	4
6	1.33	1.52	..	..
8	1.22	1.46	..	..
10	1.13	1.17	1.43	..
12	1.07	1.14	1.40	..
14	1.02	1.04	1.11	1.38

Localization energies for the *trans-cis* isomerizations in C_nH_{n+2} (the double bonds are numbered from the center of the molecule towards the end) in β units.

Consequently, it did not seem useful to take into account this refinement, which would complicate appreciably the calculations.

The results in Table 2 indicate (1) that the localization energies for the *trans-cis* isomerization are generally smaller for the internal bonds of the polyenes than for the terminal ones, with the smallest value corresponding to the central bond, and (2) that they decrease for the corresponding bonds in homologs with the lengthening of the conjugated chain. It may be remarked that the results relative to the ease of the *trans-cis* isomerizations are quite different from those relative to the stability of the corresponding hindered *cis*-isomers.

It can be shown by using the well-known expression of the L.C.A.O. method for the energy as a function of the bond orders and charges[14] that the following relation exists between the L.E.I. and the bond order $p_{\mu\nu}$ of the bond which undergoes the *trans-cis* isomerization:

$$\text{L.E.I.} = 2\beta\{p_{\mu\nu} + \sum_{r<s\neq\mu,\nu} (p_{rs} - p'_{rs}) \qquad (1)$$

where p_{rs} is the value of the bond order of the bond rs in the original molecule and p'_{rs} its value in the radical fragment. This relation is an exact one; if one would apply a perturbation treatment as is done frequently in the case of the localization energies used in the theory of chemical reactivity, one would find the approximative relation:

$$\text{L.E.I.} \simeq 2p_{\mu\nu}\beta - \beta^2 \sum_{r<s\neq\mu,\nu} \pi_{rs,\mu\nu} \qquad (2)$$

where $\pi_{rs,\mu\nu}$ is the mutual polarizability of the bonds rs, $\mu\nu$.

Relation (1) shows that the main term in the value of the localization energy for isomerizations comes from the bond order of the susceptible bond. As is well known,[14] the central "double" bonds of the polyenes have the smallest bond order, so that our result gives support to earlier statements that it is the central double bond of an all-*trans* carotenoid which should isomerize the most easily.[17, 18]

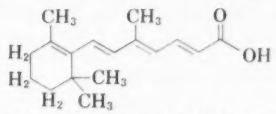
It must, however, be borne in mind that there is a second term in equation (1), so that the correlation between L.E.I. and bond order is not a perfect one. It is true, however, that the second term is small in comparison to the first one, especially as the summation generally involves alternate positive and negative terms of comparable order of magnitude. Nevertheless, this second term may be of importance in some cases, especially when one compares bonds in different or substituted molecules.

The Cis-Trans Isomerism in Retinene.—Let us now turn again to the retinene molecule. The evaluation of the L.E.I.'s around the double bonds 9–10, 11–12, and 13–14 of this molecule gives values respectively of 1.09β, 1.15β and 1.30β. (The corresponding bond orders are 0.696, 0.724, and 0.754.)[20] Consequently, these results indicate, in probable agreement with experiment 25, that the isomerization of the all-*trans* retinene to the 11-*cis* compound should be easier than the isomerization to the 13-*cis* compound. However, these values also predict that the 9-*cis* isomer should be the most easily formed, while the experimental results point, as already stated, in favor of the 11-*cis* compound.[6]

There may be two possible reasons for this discrepancy:

(1) It has been shown[21] by X-ray analysis of the crystal structure of the all-*trans*

β-ionilydenecrotonic acid (II), which is the nearest lower homolog of the acid corresponding to retinene, that the length of the single bond adjacent to the ring (6–7 bond) is 1,499 Å, practically equal to the length of the single C–C bonds in the ring (1,500 Å for bond 4–5, 1,502 Å for bond 3–4, and 1,493 Å for bond 2–3) and appreciably greater than that of any of the single bonds of the chain. This



(II) *β-ionylidenecrotonic acid (all-trans)*

seems to indicate that there can be no full conjugation across that bond, so that, to take an extreme point of view, the conjugated system is constituted essentially of the four double bonds of the chain. If the same thing occurs in all-*trans* retinene, the conjugated system in this compound would be closer to five than to six double bonds. In such a system, the 11–12 bond would become the central one and might thus be expected to have the lowest L.E.I. This explanation seems to find support in recent refined calculations on the retinene molecule[22] by a self-consistent scheme, taking into account the differences in bond lengths as given by Eichhorn's measurements. In these calculations, the bond order of the 11–12 bond is found to be effectively slightly smaller than that of the 9–10 bond, thus pointing in favor of an easier isomerization around it.

(2) Another factor which would provide a further reason for the 9-*cis* isomer to be less easily formed than the 11-*cis* may be the structure of the 9-*cis* isomer itself. It has been shown experimentally[6, 7] that the 9-*cis* retinene, as well as the related 9,13-di-*cis* compound (iso-*a*- and iso-*b*-retinenes), behave in a special way with regard to isomerization by light, as compared to the set of the other four isomers, specifically, that while the last four molecules reach the equilibrium mixture in

s-trans, all-*trans retinene* *s-cis*, 9-*cis retinene*

FIG. 1.—Hypothetical structures of the all-*trans* and the iso-*a*-compounds involving *s-cis*, *s-trans* isomerization around the bond adjacent to the β-ionone ring.

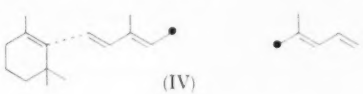
comparable times, the iso-compounds need a much longer delay. Hubbard[7] has shown that the 9, 13-di-*cis* compound isomerizes quickly to the 9-*cis* one, which then very slowly reaches the equilibrium mixture. This difference in stability between the iso-compounds and the other isomers has been puzzling enough to allow a rather bold hypothesis about their structure,[23] namely, that they may involve an α-ionone ring instead of a β-ionone one. This supposition has been abandoned, but another possibility appears, which has in fact been suggested by Dartnall:[24] it is possible that the iso-compounds involve an *s-cis* form of the retinene molecule around bond 6–7, instead of an *s-trans* form which seems to be present in the other isomers (Fig. 1).

This suggestion is supported by the recent X-ray analysis of the structure of β-ionylidene crotonic acid.[21] The authors give the *s-trans* configuration for the *all-trans* compound and the reversed *s-cis* configuration for the 9-*cis* compound. (The detailed structure has not, however, been published yet.)

This hypothesis would explain the unusual stability of the iso-compounds once formed, because in the *s-cis* configuration of the β-ionone ring, the steric interference between the methyl group at carbon 5 and the rest of the conjugated chain would be less pronounced than that caused by the *gem*-dimethyl group in the *s-trans* form, thus allowing a better conjugation in the whole molecule and enhancing consequently its stability. Furthermore, it would explain also why the 9-*cis* compound forms less easily than the 11-*cis* on irradiation of the all-*trans* as the isomerization would involve in this case not only the 9–10 double bond but also the rotation of the hexylidene cycle, thus increasing the activation energy for the overall transformation.

An interesting point is that our representation of the activated complex in the course of the isomerization may allow us to understand why the 9-*cis* isomer may have greater tendency to exist in the *s-cis* configuration than the other isomers. Let us consider the activated complexes for the 9-*cis* (III) and the 11-*cis* (IV) isomerizations and let us cut entirely the conjugation in III and IV between the

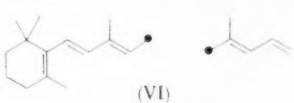
(III)



(IV)

ring and the chain as *an extreme representation* of the consequence of the lengthening of bond 6–7 beyond the usual norm (as discussed earlier). If an *s-trans–s-cis* isomerization should occur in these activated complexes, the conjugation would be restored to a certain extent between the ring and the chain (as discussed earlier). Imagining the extreme situation again, let us consider that it is then fully restored as in V and VI. Under such assumptions, we can take as a relative measure of the gain in energy which accompanies the rotation of the cycle the gain in resonance

(V)



(VI)

energy between the completely nonconjugated structures III or IV and the fully conjugated structures V or VI. The calculations have been made for the three mono-*cis* isomers: 9-*cis*, 11-*cis*, and 13-*cis* of retinene. The values obtained are respectively 0.64β, 0.59β, and 0.57β. In the present case, this gain in resonance energy is also a good indication of the probability of the isomerization because the activation energy of isomerization around that particular single bond is fairly the same for all isomers. Thus, the preceeding results show that the probability of the *s* isomerization is appreciably larger for the 9-*cis* compound than for the other two.

Conclusion.—This investigation points thus strongly toward the conclusion that it is most probably the energy of activation for the all-*trans* to *cis* isomerization rather than the relative stability of the *cis*-isomers that is the predominant factor in deter-

mining the biochemical importance of the different *cis*-retinenes.

This conclusion does not prejudge, of course, the importance of a number of complementary factors, among which, in particular, the geometrical shape of these isomers and, as pointed out recently,[22] perhaps also a number of their physicochemical characteristics such as their electron-donor ability, the basicity of their terminal carbonyl grouping, the distribution of electronic charges, etc.

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Isomer	Highest filled orbital	Lowest empty orbital
all-*trans*	−9.40	0.90
neo-*b*	−9.40	0.90
iso-*a*	−9.40	0.90
neo-*a*	−9.43	0.88
neo-*c*	−9.43	0.88
iso-*b*	−9.43	0.88

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ON THE PRESERVATION OF ANTIGENIC DETERMINANTS DURING FIXATION AND EMBEDDING FOR ELECTRON MICROSCOPY

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The study of the fine structure of cells and cellular bodies by electron microscopy has made great strides forward in the last decade. An important contribution to further development would be specific electron stains with which to identify and localize individual molecules within the cellular matrix. The success achieved by the Coons' fluorescent antibody conjugate technique of staining for light microscopy[1] clearly suggests the use of antibody as the basis for a general and highly specific electron stain. The preparation of two different types of antibody conjugate for electron microscopy has recently been reported.[2, 3]

In order to make effective and general use of an antibody conjugate as an electron stain, however, serious problems of specimen preparation must be overcome. Antibody molecules do not ordinarily penetrate living cells, and cells must therefore be first subjected to suitable treatment. Two general procedures for staining with an immuno-specific electron stain are possible. The cells might first be fixed in a manner appropriate for electron microscopy, which simultaneously renders them permeable to antibody molecules, and then stained with the antibody conjugate, followed by conventional embedding and sectioning of the cells for electron microscopy; or the cells might first be fixed, embedded, and sectioned and then stained. The latter procedure would probably be the most generally useful if it could be developed. The question immediately arises, however, as to whether antigenic determinants in a cell can be subjected to the treatment involved in fixation and embedding and still retain their capacity to bind specific antibody.

Several methods of fixation and embedding for electron microscopy are in use at the present time. In these preliminary studies, our objective was to determine the effect of some of the simpler and more widely used methods on the antigens of several simple systems. To our knowledge, no reports of such studies have yet appeared. The techniques of fixation and embedding ordinarily utilized with the fluorescent antibody conjugate method,[1] which of course do permit the retention of the antibody-binding capacity of many different antigens, are not generally applicable to electron microscopy and are therefore not pertinent in this connection.

The major part of these studies involved the antigens of *Escherichia coli* and *Streptococcus pyogenes* These organisms were chosen because they are morphologically distinct, and the specificity of staining was therefore easily ascertained in mixtures of the two. By using the fluorescent antibody technique, it was possible

to examine them rapidly and directly with the fluorescence microscope and to determine the effect of fixation and embedding treatments on the antigenic determinants of the two bacteria.

Materials and Methods.—The crystalline proteins bovine pancreatic ribonuclease (RNase) and bovine serum albumin (BSA) were obtained from Armour and Pentex, respectively. The tobacco mosaic virus (TMV) and the cultures of *E. coli* B and of a group-A, Lancefield-type-4 streptococcus were kindly furnished by colleagues at the University of California. Brain heart infusion broth (Difco) and Todd-Hewitt broth (Difco) were used for cultivating *E. coli* and *S. pyogenes*, respectively.

The γ-globulin fractions of rabbit antisera to RNase and BSA were similar to those used in other studies;[4] rabbit antiserum to TMV was part of a preparation by Bradish and Crawford.[5] The γ-globulin fraction of this serum was obtained by precipitation with 40 per cent saturated $(NH_4)_2SO_4$ adjusted to pH 6.8.

For preparation of the streptococcus immune sera, cells from a 48-hour culture were resuspended in 0.85 per cent saline containing 0.4 per cent formalin. Rabbits were given 12 intravenous injections over a three-week period and bled in the fourth week. A similar procedure was used to prepare antisera to formalinized *E. coli*.

Osmic acid fixative at pH 7.3[6] and permanganate fixative[7] were prepared as recommended. Embedding was carried out in a methacrylate monomer mixture by three methods: (1) thermal polymerization at 50° C. for 10 hrs, (2) thermal polymerization at room temperature for 2 days in the presence of an accelerator, or (3) ultraviolet-induced polymerization at -10°C for 10 hrs in the presence of a photosensitizer. The monomer mixture in each case consisted of three parts of butyl methacrylate to one of methyl methacrylate and contained one per cent benzoyl peroxide. In addition, to this mixture was added either 0.5 per cent dimethylaniline for method (2) or 0.2 per cent benzoin for method (3). For the ultraviolet irradiation, a Westinghouse Black Light Blue fluorescent lamp, F15T8/BLB, was used in a cold room at -10°C.

To determine the effects of the fixatives on the soluble antigens RNase, BSA, and TMV, 0.10 ml. of a concentrated protein solution was mixed with 2.0 ml. of fixative at 0°C for 30 min. The solutions were then dialyzed overnight at 4°C against 3 liters of phosphate buffer, pH 7.5, $\Gamma/2$ 0.1. If these solutions contained insoluble matter, they were then centrifuged to remove it. A portion of this solution was then mixed at 37°C with a solution of γ-globulin containing homologous antibody, and the flocculation time and the amount of precipitate formed was compared with a control containing the same amount of untreated antigen and homologous antibody, and with another control containing the treated antigen and normal γ-globulin.

The bacterial cells were fixed and embedded by the following procedure. Equal volumes (300 ml or less) of overnight cultures of the two bacteria were mixed, centrifuged, washed once with 40 ml of phosphate buffered saline (PBS) at pH 7.0, centrifuged again, and resuspended in 5 ml osmic acid fixative. After fixation at 0°C for 10 minutes, the mixture was diluted with 35 ml of cold PBS and the cells were centrifuged down. During fixation, the cells formed into clumps, which proved to be undesirable for purposes of specific staining; these clumps were therefore broken up by resuspending the cells in PBS and drawing them in and out of a syringe fitted with a 20-gauge needle until microscopic examination showed most of the cells to be singly dispersed. The PBS wash was repeated twice using 40 ml volumes at 0° C.

The cells were then washed successively for 10–20 minutes at room temperature once with 15 ml each of 25%, 50%, 75% ethanol, twice with 15 ml of absolute ethanol, once with 5 ml of a 1:1 mixture of methyl methacrylate and absolute ethanol, once with 5 ml of methyl methacrylate, and twice with 5 ml of a 3:1 mixture of butyl and methyl methacrylates. The cells were divided at this point into three portions and resuspended in the respective polymerizing mixtures used for the three polymerization methods discussed above. After transfer to gelatin capsules, they were centrifuged, the polymerizing mixture was replaced, and embedding was carried out.

Fluorescence microscopy was performed with a Zeiss fluorescence microscope illuminated with an Osram HBO 200 mercury vapor burner. The filter system consisted of the UG 2 and UG 5 exciter filters and the GG 4 barrier filter. A monocular microscope equipped with a cardioid darkfield condenser and a 40X oil immersion fluorite objective was used. Fluorescent antibody conjugates were prepared with fluorescein isothiocyanate and used in the direct staining method

described by Coons and Kaplan.[8] Those labeled antibody preparations that stained nonspecifically were first absorbed with the heterologous organisms.

Smears were prepared from cells after each of the various steps of fixation, dehydration, and embedding and were then stained. With the embedded cells, the intense blue autofluorescence of polymerized methacrylate made it necessary to dissolve the polymer away from the cells before staining. This was accomplished by exposing the embedded sections to benzene overnight at 4°C, followed by washes with benzene-ethanol, ethanol, and PBS. Smears of these cells were then made and stained. The intensity of staining was arbitrarily graded in 5 steps from ++++ to −, each step corresponding to about a 25 per cent decrease in intensity of fluorescence. Staining specificity was confirmed by (1) staining mixtures of the two bacteria which could be distinguished morphologically, (2) inhibiting staining with unlabeled antibodies,[9] and (3) removal of labeled antibodies by the addition of homologous bacterial suspensions.

Results.—Treatment of the soluble antigens RNase, BSA, or TMV with osmic acid fixative under the conditions described produced no change of color of the protein solution nor any other evidence of chemical reaction. The flocculation time and the amount of specific precipitation by antibody were unaffected. On the other hand, RNase and BSA solutions treated with $KMnO_4$ fixative acquired a deep brown color but remained soluble, while a similarly treated TMV solution showed a copious brown precipitate. None of these proteins gave any visible precipitate when treated with their homologous antibodies.

Osmic acid fixation of previously dried smears of the bacteria resulted in a high degree of nonspecific staining. If, however, the cells were fixed in suspension instead of in smears and washed in suspension with PBS by the methods described above, the intensity and specificity of staining were the equivalent of those found with ethanol-fixed cells. Osmic acid-fixed cells which were dehydrated, embedded in methacrylate, and treated as described to remove the methacrylate polymer, stained with a +++ intensity compared to ++++ for the cells which were fixed only. This was true for both types of cells. This drop in staining intensity was a gradual one occurring through the course of the procedure rather than at any one step in it. The specificity of staining was found to be maintained throughout the procedure, as tested by all three methods described in the previous section, and is illustrated in Figure 1. There was no observable difference in staining produced by the three different methacrylate embedding methods used.

Discussion.—Several methods of fixation for electron microscopy have been developed. Fixation by rapid freezing, followed by sublimation of the frozen water at very low temperatures[10] or followed by substitution of the frozen water by a polymerizable monomer at very low temperatures,[11] would appear to be the method least likely to inactivate antigenic determinants. However, these are still cumbersome and time-consuming methods, and chemical fixatives are more widely used. We have shown that treatment with osmic acid fixative has no serious effect on the capacity of several different types of antigens to bind specific antibody. Our results suggest further that protein antigens generally will be found to be unaffected by osmic acid, since there was no indication that any chemical reaction had occurred with the several protein antigens examined. It is therefore quite likely that osmic acid fixation will be a generally useful technique in preparing specimens for staining with antibody conjugates for electron microscopy. If other types of antigen systems are encountered which are inactivated by such treatment, resort may be had to rapid freezing methods.

On the other hand, $KMnO_4$ fixative completely inactivated these same protein antigens, which is not too surprising in view of the powerful oxidizing capacity of $KMnO_4$. It is clear therefore that this fixative is not useful for our purposes.

Embedding of cells and tissues for electron microscopy has been carried out successfully and reproducibly in a variety of polymerizing systems. In many of these, the monomers contain reactive chemical groups which can attack groups in the antigenic sites of proteins and other macromolecules. For example, the epoxide groups of the Araldite embedding system[12] are likely to react with amino and other groups of antigenic components[13] and possibly inactivate them in the process.[14] For these preliminary experiments, therefore, we confined ourselves to methacrylate embedding, since in a vinyl polymerization the small concentration of free radicals and their selective reactivity for monomer molecules were expected to favor retention of antigenic activities. It was not possible to stain sections of the embedded cells directly with fluorescent Ab because of the intense autofluorescence of the polymerized methacrylate; instead, the stain was applied after the polymer was dissolved away from the cells. This permitted us to determine whether the cells had retained their antigenic activities during the embedding process, but no information was obtained as to whether cells within the embedded matrix could be stained. We did indeed find that polymethacrylate embedding, even if carried out at 50°C, permitted the retention of at least a substantial fraction of the antibody-binding capacity of the osmic acid-fixed bacteria.

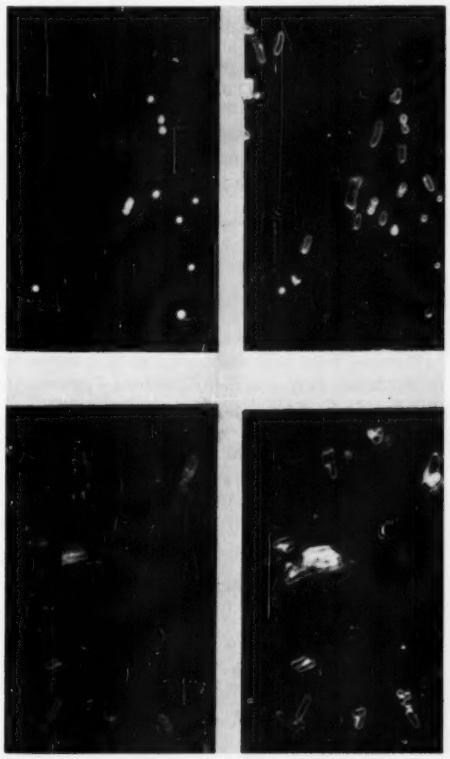

FIG. 1.—Photomicrographs of mixtures of *E. coli* and *S. pyogenes* which were fixed in osmic acid and embedded in methacrylate as described in the text. The two top pictures are of a specimen which was stained with fluorescein-labeled anti-*S. pyogenes* antibodies, and examined in visible light (top right) and in the ultraviolet (top left). The spherical *S. pyogenes* cells were stained specifically. The two bottom pictures are of a specimen similarly prepared, but stained with fluorescein-labeled anti-*E. coli* antibodies, and examined in visible light (bottom right) and in the ultraviolet (bottom left). At least 10 well resolved *S. pyogenes* cells may be observed to be not stained, and the rod-like *E. coli* cells were therefore stained specifically.

These results are too few to permit any generalizations to be made about the preservation of a wide variety of antigenic determinants through osmic acid fixation and methacrylate embedding. This is particularly so because the antigens of

bacteria are many and complex,[15] and it is not evident whether they are more or less stable to inactivation than viral or other types of antigens. Nevertheless, these results do strike an optimistic note for further investigations. Furthermore, these studies have provided us with a model system of antigenically-active and suitably embedded bacterial cells with which to test various antibody conjugates as specific stains for electron microscopy, and such investigations are in progress.

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* John Simon Guggenheim Memorial Foundation Fellow, 1959–60, on leave from Yale University.

† Contribution No. 1641.

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STUDIES ON THE COMPOSITION OF THE PROTEIN FROM ESCHERICHIA COLI RIBOSOMES

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Much recent evidence indicates that protein synthesis takes place on the ribonucleoprotein particles now commonly known as ribosomes. Both *in-vitro* and *in-vivo* experiments with radioactive amino acids have produced evidence that peptide linkages are formed on ribosomes and that the growing polypeptide chains remain attached to ribosomes until their completion. A question thus poses itself: What fraction of the total protein of the ribosomes consists of growing or newly

completed polypeptide chains, and what is the chemical nature of the remainder of the ribosomal protein, which may be regarded as a structural component of the ribosomes? Calculations from the amount of bound amino acids in *in-vitro* experiments suggest that the growing peptide chains represent approximately 0.1 per cent of the total ribosomal protein.[1, 2] This figure, however, might seriously underestimate newly made protein, if some does not immediately leave the ribosomes but remains attached for an indefinite time. In the work presented here, this problem is directly attacked by examining *E. coli* ribosomal protein for its amino-terminal groups. Only two major end groups are found, methionine and alanine. Ribosomal protein thus appears not to be a random sample of total cellular protein but on the contrary, a class (see below) of basic proteins which quite possibly serves the role of maintaining ribosomal RNA in a suitable configuration for protein synthesis.

There exist four main varieties of *E. coli* ribosomes, with sedimentation constants of 30 S, 50 S, 70 S, and 100 S.[3–5] Each type contains about 63 per cent RNA and 37 per cent protein. At suitable magnesium ion concentration, one 30 S and one 50 S ribosome combine to form a 70 S ribosome, while the 100 S ribosome is a dimer of two 70 S ribosomes which forms at higher magnesium ion concentration. We have examined the amino end groups of the protein from both 70 S ribosomes and its 30 S and 50 S subunits by the fluorodinitrobenzene (FDNB) method of Sanger.[6]

Preparation of Ribosomal Proteins.—The three types of particles were prepared as previously described.[3] The protein fractions which were used for NH_2-terminal group analysis were prepared by the acetic acid procedure.[7] Two volumes of cold glacial acetic acid were added to a cold solution of ribosomes (1–3% by weight), and the RNA which immediately precipitated was kept in the form of a fine suspension in a teflon homogenizer. After 45 minutes at 2–4°, the RNA precipitate was collected by centrifugation; the supernatant solution was dialyzed in the cold to remove most of the acetic acid (as well as any free amino acids or small peptides which may have been associated with the ribosomes) and freeze-dried in order to recover the protein. In this manner, protein preparations containing about 85 per cent of the total protein of the ribosomes were obtained. The remainder of the protein was retained in the RNA precipitate and could not be extracted from it even after repeated washing with 67 per cent acetic acid. This protein fraction has since been separately isolated by another method (J.-P. Waller, unpublished data), and preliminary studies indicate that it is essentially similar in end groups, and not greatly dissimilar in amino acid composition, to the main acetic acid–soluble protein fraction.

NH_2-Terminal Group Analysis.—The protein extracted with 67 per cent acetic acid showed a marked tendency to aggregate, with resulting insolubility over a wide range of pH. Coupling reactions with FDNB reagent[6, 8] were accordingly carried out in the presence of either 8 *M* urea or 6 *M* guanidine hydrochloride. In most cases, the protein (20 mg in 1 per cent solution) was allowed to react with FDNB in 0.1 *M* $NaHCO_3$ containing 8 *M* urea (pH 8.7) at 40° for four hours. The DNP-protein was precipitated with HCl, collected by centrifugation, and washed successively with *N* HCl, acetone, and ether. It was then hydrolyzed in a sealed evacuated tube with constant boiling HCl for 12 hours at 110°, and the DNP-

derivatives of its NH_2-terminal amino acids were identified and estimated according to the method of Levy.[8] Special hydrolyses were performed for the estimation of DNP-proline and DNP-glycine.

Results.—The results obtained in a series of experiments with protein preparations from 70 S, 50 S, and 30 S ribosomes are summarized in Table 1. In all cases, alanine and methionine, the latter in slight excess, were found to be the major NH_2-terminal amino acids, and collectively they account for about 85 per cent of the total end groups found. Smaller amounts of DNP-serine and threonine and traces of DNP-aspartic and glutamic acids were also found. Although these occurred in relatively minor amounts, they were nevertheless obtained consistently and in reproducible proportions from several different preparations of 70 S ribosomes. The fact that they also occur in the proteins from both 30 S and 50 S ribosomes suggests very strongly that they represent the NH_2-terminal groups of genuine protein chains in the ribosomes. These results, in agreement with the results of quantitative amino acid analyses,[9] also reveal a remarkable similarity between the protein moieties of the three types of nucleoprotein particles.

The possibility that the minor end groups arise through degradation of the protein, either during the isolation procedure or during its subsequent reaction with FDNB, has been considered, but the results of suitably designed control experiments do not support this view. When, for example, ribosomal protein was isolated by a procedure[10] which involves the use of 4 *M* urea at pH 7.3 and 20°, the minor end groups were found to occur in the same proportions as had been found for the protein prepared by the acetic acid procedure. There was no increase in the amounts of minor end groups when the FDNB reaction was carried out in 8 *M* urea at pH 8.7 and 40° for 4 hours (i.e., standard conditions), or 12 hours, respectively. The same end group values were also obtained when the FDNB reaction was carried out in 6 *M* guanidine hydrochloride at pH 7.3 and room temperature for 5 hours or 12 hours respectively.

When appropriate corrections are applied to account for hydrolytic and chromatographic losses incurred during the DNP procedure, a value of about 25,000 may be calculated for the *average* molecular weight of the constituent protein chains in ribosomes. The calculation is based on the recoveries of the two major end groups, which were estimated by hydrolyzing the appropriate DNP-amino acid in the presence of DNP-protein (from 70 S ribosomes). For DNP-alanine and DNP-methionine (including methionine sulfoxide), the mean recovery values for two determinations were 77.1 (±0.8) per cent and 79.3 (±1.8) per cent respectively. Recoveries of the minor groups were not estimated, but they should not significantly alter the results in Table 1.

In arriving at the average molecular weight value of 25,000, we have made the following assumptions: (*a*) that all the protein chains possess NH_2-terminal amino acids which are free to react with FDNB, and (*b*) that the DNP-amino acids which we have identified and estimated represent genuine NH_2-terminal residues of the protein. Further work involving a much more comprehensive analysis of the protein is being undertaken to establish the validity of these assumptions.

The results obtained by the FDNB method were confirmed when the paper strip modification[8] of the phenylisothiocyanate method[11] was applied to ribosomal protein. In this manner, alanine and methionine were again found to be the major

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The proportions of the NH_2 terminal groups are expressed as percentages of the total end-groups found. Results not corrected for hydrolytic and chromatographic losses (see text).

Particle preparation*	70 S (C)	70 S (C)†	70 S (D)	70 S (E)‡		50 S (E)‡		30 S (E)‡	
NH_2-terminal amino acid:									
Alanine	37.9	36.3	37.2	36.1	36.1	40.0	38.2	36.0	37.0
Methionine	48.5	45.4	49.2	46.7	46.0	47.2	48.7	46.6	45.8
Serine,§	8.8	13.0	8.2	11.4	11.6	10.7	10.2	12.6	12.3
Threonine	2.9	3.9	3.2	3.9	3.8	1.1	1.8	3.1	3.2
Aspartic acid and glutamic acid	1.8	1.3	1.0	1.1	1.5	0.9	1.1	1.7	1.6
Lysine	trace	trace	1.1	0.9	1.0	—	—	—	—
Weight of protein/mole of end groups (uncorrected)	35,600	30,200	33,200	32,400	31,400	32,100	31,900	39,600**	43,200**
Weight of protein/mole of end groups (corrected)††	27,800	23,600	25,900	25,300	24,500	25,000	24,900		

* Letters C, D, and E refer to particles obtained from different *E. coli* preparations.

† Reaction in the presence of 6 *M* guanidine-HCl and $KHCO_3$ (pH 7.3) for 5 hours at 40°. All other reactions in 8 *M* urea and $NaHCO_3$ (pH 8.7) for 4 hours at 40°.

‡ Each sample analyzed in duplicate. The 50 S particle preparation used was virtually pure, while the 30 S preparation was contaminated by approximately 20% of 50 S.

§ Values uncorrected for minor contamination by methionine sulfoxide. By rechromatography of the DNP-"serine" spot in the solvent benzene–acetic acid–water (1:1:1), the average serine value for protein from 70 S particles was established as 8% of the total end groups. Higher values were accompanied by a corresponding decrease of the methionine and can be attributed to methionine sulfoxide.

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end groups, while serine, threonine, and aspartic and glutamic acids were also present in smaller amounts. The average molecular weight of the protein chains (calculated from the optical density at 268 mμ of the phenylthiohydantoin deriva-

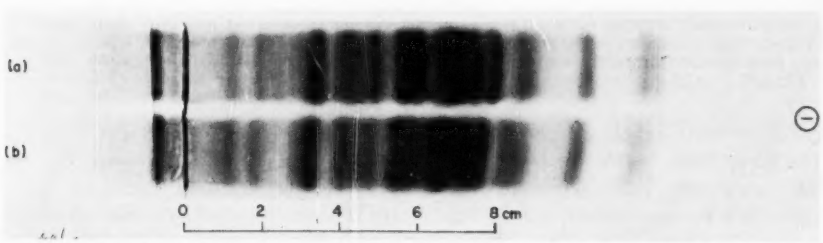

FIG. 1.—Starch gel electrophoresis of *E. coli* ribosomal proteins in the presence of 6 *M* urea (0.0365 *M* sodium acetate buffer; pH 5.6 within the gel; length of run 24 hours at 9 v/cm; temperature within the gel 8–10°). (*a*) is acetic acid soluble protein (3.5 mg.); (*b*) is *E. coli* ribosomal particles (7.5 mg) dissolved in 6 *M* urea, pH 5.6 buffer. That the acetic acid soluble protein is well representative of the total ribosomal protein is apparent from a comparison of the two band patterns.

tives of the NH_2-terminal amino acids) was found to be 26,000, a value in good agreement with that obtained by the FDNB method.

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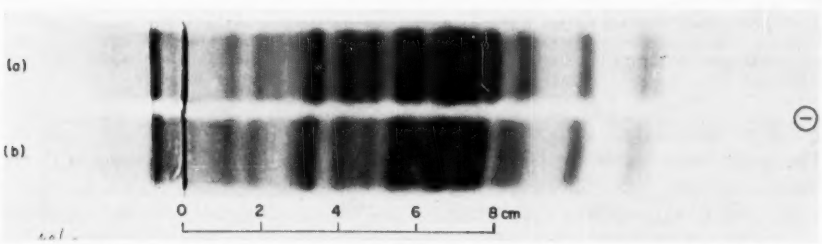

FIG. 1.—Starch gel electrophoresis of *E. coli* ribosomal proteins in the presence of 6 *M* urea (0.0365 *M* sodium acetate buffer; pH 5.6 within the gel; length of run 24 hours at 9 v/cm; temperature within the gel 8–10°). (*a*) is acetic acid soluble protein (3.5 mg.); (*b*) is *E. coli* ribosomal particles (7.5 mg) dissolved in 6 *M* urea, pH 5.6 buffer. That the acetic acid soluble protein is well representative of the total ribosomal protein is apparent from a comparison of the two band patterns.

Fractionation of the protein from E. coli ribosomes (70 S): In order to further investigate the composition of *E. coli* ribosomal protein in the light of the NH_2-end

group results, the protein has been submitted to various fractionation procedures, including starch gel electrophoresis[12] and ion-exchange chromatography.

When acetic acid–soluble protein from ribosomes or intact 70 S ribosomes are submitted to starch gel electrophoresis in the presence of 6 *M* urea at pH 5.6, a complex pattern of at least 20 bands appears on staining with amido black (Fig. 1). The possibility that aggregation may be responsible for the complexity of this pattern has been considered, but it appears most unlikely on the basis of the following results:

(*a*) When acetic acid–soluble protein, adsorbed on a column of carboxymethylcellulose[13] in a solution of 0.01 *N* HCl in 6*M* urea (pH 3.32) at 2° is eluted by progressively lowering the pH to 2.50, the protein can be recovered in two distinct chromatographic peaks, I and II. Starch gel electrophoresis in 6 *M* urea at pH 5.6 of the material from the two peaks (recovered after extensive dialysis against water and freeze-drying) shows a separation into two distinct sets of bands, which to-

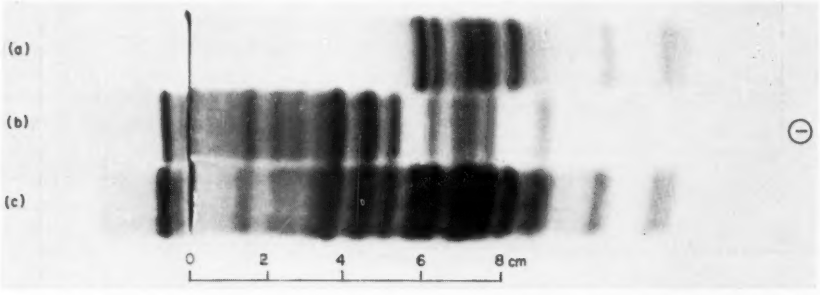

FIG. 2.—Starch gel electrophoresis in 6 *M* urea at pH 5.6 (for conditions, consult Fig. 1) of the materials from peaks I and II obtained when acetic acid-soluble protein is fractionated on carboxymethylcellulose under conditions described in the text. (*a*) is protein from peak II (2.5 mg); (*b*) is protein from peak I (2.5 mg); (*c*) is unfractionated acetic acid–soluble protein (3. 5 mg). The bands in the overlapping region of (*a*) and (*b*) appear to be two distinct sets of bands, the combination of which accounts for the intense staining in the same area of the unfractionated protein. This apparent is ascribed to the influence of the carboxyl groups of the protein. While these were only partly ionized during chromatography in acid pH, they are fully ionized during electrophoresis at pH 5.6.

gether account for all the bands observed in the unfractionated protein (Fig. 2). The more basic bands are from the material in peak II, in accordance with its chromatographic behavior on carboxymethyl-cellulose.

(*b*) As a further control, the material eluted from carboxymethyl-cellulose in the system described above was subdivided into ten arbitrary fractions from successive portions of the effluent in the order of increasing acidity. The protein from each of these fractions was isolated by extensive dialysis against water and freeze-drying and was submitted to starch gel electrophoresis in 6 *M* urea at pH 5.6. In spite of a considerable overlap of the bands from immediate neighboring fractions, a clear separation into small groups of bands, with increasingly basic properties from fraction 1 to 10, was observed. There was no apparent evidence of re-aggregation.

The proteins from peaks I and II, derived from chromatography on carboxymethyl-cellulose as described above, were separately examined for NH_2-terminal groups by the FDNB method. Alanine and methionine end groups were present in

both fractions. The results clearly indicate that in ribosomes the protein chains possessing an NH_2-terminal methionine (or alanine) residue are not all chemically identical.

Discussion and Summary.—NH_2-terminal group analyses on *E. coli* ribosomal protein indicate the presence of two major end groups: methionine and alanine. The results are consistent with the view that ribosomal protein is not a random sample of total cellular protein but, on the contrary, a class of basic proteins which quite possibly serves the role of maintaining ribosomal RNA in suitable configuration for protein synthesis.

The constituent protein chains of ribosomes, with an *average* molecular weight of 25,000, thus fall into two major classes: those chains with methionine as NH_2-terminal residue and those with alanine. From the results of fractionation experiments, it is further apparent that within each of these two major classes not all chains are chemically identical. The extent and significance of the chemical differences between chains possessing the same NH_2-terminal residue are now being investigated. It is of interest to note that, in this respect, *E. coli* ribosomal protein bears a striking resemblance to calf thymus histone, where a simple amino end group picture (with alanine and proline accounting for over 90 per cent of the end groups[14]) is likewise accompanied by a complex protein composition.[15]

We wish to thank Drs. J. T. Edsall, J. D. Watson, and, A. Tissières for their interest and for helpful discussions. We are indebted to the National Institutes of Health (Grant H-3169) and the National Science Foundation (Grants G-9116 and G-10895) for research support.

* Member of the Scientific Staff of the Medical Research Council of Great Britain and Research Fellow in Biology, Harvard University, 1959.

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THE ESSENTIALITY OF COBALT FOR SOYBEAN PLANTS GROWN UNDER SYMBIOTIC CONDITIONS

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The biological importance of cobalt was first recognized by the discovery that small amounts of the element would cure certain deficiency symptoms in sheep and cattle.[1–3] A careful review of the sequence of events leading to these findings has been provided by Underwood.[4] After the early work, it has become apparent that a major role of cobalt in the metabolism of animals and microorganisms is associated with the necessity of the element as a constituent of vitamin B_{12}.[5, 6] The original research with ruminants has stimulated many investigations,[7–9] revealing that many microorganisms require preformed vitamin B_{12} whereas others synthesize the vitamin if cobalt is available. Of special interest is a report[9] that certain blue-green algae are dependent upon either cobalt or vitamin B_{12} for normal growth and that those species that fix atmospheric nitrogen exhibit a greater response than those requiring a source of fixed nitrogen. It is generally recognized that vitamin B_{12} is synthesized only by microorganisms. The vitamin has been identified in root nodules of leguminous plants[10] and in roots of nonleguminous species,[8] but in these cases the synthesis has been attributed to the nitrogen-fixing bacteria in nodules and to soil microorganisms respectively.

Even though cobalt has been recognized as a micronutrient for animals and certain microorganisms, until recently there has been no conclusive evidence of its essentiality for higher plants. The efforts of Hewitt and Bolle-Jones[11] to demonstrate a cobalt requirement for tomato, lettuce, and sugar cane failed to produce positive results. Bolle-Jones and Mallikarjuneswara,[12] however, have shown that the addition of small amounts of cobalt to cultures of tomato and rubber plants supplied with purified nutrient solutions resulted in significant increases in dry weight, stem girth, and plant height. The lack of cobalt failed to produce visual symptoms of deficiency or consistent differences in the content of chlorophyll or of the major essential elements. These workers, therefore, did not claim cobalt to be an essential mineral element.

In view of these observations, the authors initiated experiments designed to determine whether or not leguminous plants grown under conditions which forced them to depend upon atmospheric nitrogen required cobalt for normal growth. Results of the early experiments[13] showed that the addition of cobalt produced a marked increase in the dry weight of soybean shoots and prevented the development of nitrogen-deficiency symptoms that were apparent on plants that were not provided with the element. After this, Reisenauer[14] demonstrated that alfalfa plants grown in nutrient solutions lacking added cobalt and containing a limited nitrogen supply developed marked nitrogen-deficiency symptoms and produced 49 per cent less dry weight than plants supplied with the element. Also, the addition of cobalt to cultures of alfalfa resulted in a striking increase in the total nitrogen content of the plant material. A stimulatory effect of cobalt and copper on nitrogen fixa-

tion by subterranean clover has been reported by Hallsworth, *et al.*[15] Further refinement in the methods of culture and purification by the authors has resulted in the conclusive demonstration that cobalt is an essential element for the growth of soybean plants under symbiotic conditions. A summary of some of these results has already been published.[16] It is the purpose of this paper to provide the detailed evidence of the effect of cobalt on the growth and chemical composition of symbiotically grown soybean plants. Evidence also is provided that soybean plants grown in purified nutrient solutions with adequate fixed nitrogen failed to respond to added cobalt.

Methods and Materials.—The methods used for the purification and storage, of water, mineral salts, and stock solutions unless otherwise indicated were the same as those described or referred to in a previous publication.[16] For the purification of all the macronutrient salts and $MnSO_4 \cdot 4H_2O$, the 1-nitroso-2-naphthol extraction procedure of Bolle-Jones and Mallikarjuneswara[12] was used. In this procedure, 1.5 liters of stock solutions (0.5 *M*) of the salts were warmed to about 45°C and treated with approximately 10 ml of 1-nitroso-2-naphthol (0.12 per cent in 0.05 per cent aqueous NaOH). After cooling, 750-ml portions of each salt solution were extracted with 50–60 ml of redistilled chloroform to remove cobalt and other metal complexes and the excess reagent. The extractions were repeated until no color was observed in the chloroform phase. Ordinarily, three or four extractions were sufficient. The last traces of the reagent and the chloroform still remaining in the salt solution were removed by addition of activated charcoal (previously washed with 3 *N* redistilled HCl). For each 1.5 liters of the solution, 0.75 to 1 gm of activated charcoal was added. The solution was heated and allowed to cool. The solutions were filtered by using acid-washed filter papers, and the filtrates were stored in acid-washed polyethylene bottles.

Ferric citrate was used as the source of iron, and calcium hydroxide was used to adjust the pH of nutrient solutions. These compounds and the micronutrient additions were prepared by the methods described previously.[16] In the experiment (Table 1) where V or a mixture of Ni, Ga, and Al was added in order to determine whether or not these elements would replace cobalt, spectrographically standardized† or twice crystallized compounds were utilized as sources of the elements.[16] Cobalt was added to all cultures as $CoCl_2$ prepared by dissolving spectrographically standardized metal† in redistilled HCl. In one test, Ru, W, Pd, Ag, Sn, Bi, U, and Cr each at one ppb‡ was supplied to cobalt deficient plants as a mixed treatment. These elements were added as the following spectrographically standardized compounds:† $(NH_4)_2RuCl_5$, WO_3 in NH_4OH, NH_4PdCl_4, AgCl in NH_4OH, SnO_2 in NH_4Cl, Bi_2O_3 in HCl, U_3O_8 in HNO_3 and K_2CrO_4. A stock solution of the mixture of these eight elements was prepared and a sufficient quantity was added to the culture to supply one part per billion (ppb)‡ of each.

The basic nutrient solution utilized for growing soybean plants under symbiotic conditions was a nitrogen-free solution containing phosphate in the forms of KH_2PO_4 and K_2HPO_4.[16] The basic culture solution utilized for growth of plants with adequate fixed nitrogen contained the following macronutrient elements (me. per l): K^+, 5; Mg^{++}, 4; Ca^{++}, 13; PO_4^{---}, 3; NO_3^-, 4.6; NH_4^+, 2.4; SO_4^{--}, 17; and the following essential micronutrient elements (ppm): Zn^{++}, 0.05; Mn^{++}, 0.15; Cu^{++}, 0.01; BO_3^{---}, 0.1; MoO_4^{--}, 0.02, Cl^-, 0.2, and Fe^{+++}, 1.0 every week.

The NO_3^- was supplied as $Ca(NO_3)_2 \cdot 4H_2O$ and NH_4^+ as $(NH_4)_2\ SO_4$, and the other elements were supplied in the forms already reported.[16] All nutrient solutions were maintained at pH values ranging between 6.5 and 7.0 by adding at 3-day intervals the required amount of $Ca(OH)_2$ suspension.

The treatments employed in the experiment where plants were grown under symbiotic conditions are outlined in Table 1, and those in the experiment where

TABLE 1

EFFECT OF COBALT AND OTHER ELEMENTS ON THE YIELD OF VARIOUS PARTS OF SOYBEAN PLANTS AND ON THE PRODUCTION OF VIABLE SEED UNDER SYMBIOTIC CONDITIONS

Treatments	Shoots*			Roots‡	Root nodules	Seed	Viable seed (No. per culture)
	Initial	1st axillary	2nd axillary†				
	Dry wt in gm per culture§						
No Co	3.0	3.4	4.4	4.0	0.7	0.4	8
1 ppb each of Ni, Al, and Ga	2.7	4.0	8.0	5.0	1.2	2.5	29
1 ppb of V	2.8	3.5	8.0	5.0	1.4	1.6	23
0.1 ppb of Co	4.5	14.6	63.3	15.0	4.4	31.0	256
1.0 ppb of Co	3.8	13.2	55.3	13.0	5.1	33.1	237
Least significant difference at 5%	0.9	3.0	9.1	4.4	2.1	15.8	26
Least significant difference at 1%	1.3	4.6	12.9	4.8	3.0	22.4	37

* The initial and 1st axillary shoots were harvested 5 and 8 weeks respectively after transferring the seedlings to the culture vessels. The final harvest of the 2nd axillary shoots, roots, root nodules, and seed was made 15 weeks after transferring seedlings to culture vessles.

† Weights of pericarps are included with shoots in the 2nd axillary growth.

‡ The weights of nodules are included with weights of roots.

§ All weights are expressed on an oven-dry basis with the exception of those for seed which are expressed on an air-dry basis. All data presented are means of determinations on four replicate cultures.

plants were grown in the presence of adequate fixed nitrogen are outlined in Table 5. In both experiments, the appropriate basic nutrient solutions were supplied to all cultures, and the treatments consisted of the addition of cobalt or other metals as outlined in Tables 1 and 5, respectively. Each individual treatment except the control (no cobalt) of the experiment outlined in Table 1 was applied to four replicate cultures of soybean plants. In the experiment involving symbiotically grown plants (Table 1), the no-cobalt treatment was applied to eight replicate cultures for the purpose of producing extra cobalt-deficient plants. The four extra cultures were used in curing and specificity experiments. Vanadium or a combination of Ni, Ga, and Al was added as a treatment in one experiment because these elements have been reported to stimulate the growth of algae, fungi, or higher plants.[17-20] The mixture of Ru, Cr, W, Pd, Ag, Sn, Bi, and U used to test the possibility of other elements replacing cobalt for the growth of soybean plants was selected because evidence in the literature[21] suggested that these elements might have been removed by the purification procedure.

The cultural methods employed and the design of the experiment carried out under symbiotic conditions were the same as those described for experiment III in a previous report.[16] Soybean seedlings *Glycine Max. Merr.* (variety Roanoke) were used in all experiments. The cotyledons of young seedlings were removed four days after transplanting in order to minimize the transfer of cobalt from the seed to the young plants. In the experiment involving symbiotically grown plants, the temperature in the growth chambers was maintained between 79° and 81°F, and the light intensity at two feet above the plants was 2,000 foot-candles. The

day length was maintained at 15 hours until three weeks after harvesting the first axillary shoots; then it was decreased to 9 hours in order to induce flowering. In the experiment where fixed nitrogen was supplied (Table 5), the four replications of the three treatments were designed in a randomized block in the growth chamber. These plants were grown in the same temperature range and at the same light intensity as the symbiotically grown plants. The day length, however, was maintained at 15 hours throughout the growth period. The other cultural methods utilized for growth of plants with adequate fixed nitrogen (Table 5) were the same as those used for the experiment involving symbiosis with the exceptions that the inoculation with *Rhizobium japonicum* was omitted and a source of fixed nitrogen as previously indicated was included in all nutrient solutions.

In both experiments, the initial shoots (see Tables 1 and 5 for growth periods) were cut back just above the third node above the cotyledons. Axillary growth of shoots harvested after the initial shoot growth or after the growth of previous axillary shoots was obtained by cutting shoot stems just above the third node on each shoot stem.

Immediately after harvesting the shoots or other plant parts, the fresh weight of the material was recorded, and the plant parts, except for the seed, were dried in an oven for 12 hours at 70°C before weighing. The seeds were dried inside the pods for eight days at a temperature of 30°C before recording the air-dry weights. At the time of final harvest, a random sample of about 10 per cent of the total roots was taken from each of the cultures, and the nodules from the samples were collected. The nodules and the root samples were oven-dried, and on the basis of the weights of these, the entire dry weight of nodules from each culture was calculated.

In order to determine the viability of the seed, all the seed obtained from each of the no-cobalt cultures and 20 seeds from each of the cultures receiving cobalt treatments were germinated between folds of paper towels that were kept moist with $^1/_{10}$ strength nutrient solution. These were incubated for eight days at 27°C, and the number of normal seedlings was recorded.

Total nitrogen content of the dry plant material was determined by a semi-micro Kjeldhal procedure,[22] and the chlorophyll content of fresh leaves was assayed by the method of Mackinney.[23] For the latter determination, a random sample of two leaflets was collected from the third trifoliate leaf below the growing point of each of four plants. The eight leaflets collected from each culture were cut into pieces about 0.5 cm in diameter and a one-gram sub-sample was used for the determination.

For the determination of hemoglobin and vitamin B_{12}, a random sample of about two grams of nodules was collected from each culture at the time of harvesting first axillary shoots. The hemoglobin content of nodules was determined on a sub-sample by the method of Hartree.[24] For the determination of vitamin B_{12}, each sample (0.5 gm) of nodules was extracted by macerating with 20 ml of 0.01 per cent KCN, and then the macerated material was allowed to incubate for 30 minutes at 60°C. After this, each sample of macerated nodules was centrifuged, and the supernatant fluid collected for the assays. The apparent vitamin B_{12} content was assayed by the *Lactobacillus leichmannii* (ATCC 7830) method,[25] and the true vitamin B_{12} content was determined using *Ochromonas malhamensis* by the method outlined by Ford.[26] The cultures were incubated at 28°C in the dark, and the

turbidities in both assays were measured in a Coleman colorimeter using a red filter.

The cobalt content of the dry material was determined on the ash of the samples by the method of Saltzman.[27] For this procedure, 9 to 34 gm of composite plant samples, depending upon the size of the sample available and the source of sample with reference to cobalt treatment, were ashed at 650°C in a muffle furnace. The ash was digested with portions of HNO_3 (diluted with an equal volume of H_2O) until all traces of dark color disappeared and then it was dissolved in dilute H_3PO_4 (1 part H_3PO_4 and 49 parts H_2O) and the procedure of pre-extraction and final development of color by nitroso-R-salt was followed.[27]

Excepting for cobalt determinations, all analyses were carried out on plant material from each of the four replicate cultures receiving a particular treatment. The data were subjected to analyses of variance as indicated in the tables.

Experiments under Symbiotic Conditions.—Effect on growth and seed production: In a series of experiments, the effect of cobalt was studied on the growth of soybean plants under symbiotic conditions. The particular experiment under consideration was conducted after final refinement of the purification and cultural techniques. The treatments employed (outlined in Table 1) included cobalt at 0.1 and 1 ppb in culture solutions, and these were compared with a treatment lacking added cobalt. Also included in the experiment were two other treatments lacking added cobalt. One of these contained vanadium at one ppb and the other contained a mixture of Al, Ga, and Ni each at one ppb. During the course of plant development it was obvious that of all of these elements tested, only cobalt at 0.1 and 1.0 ppb resulted in a pronounced beneficial effect on the growth of plants. Plants in cultures without added cobalt or supplied with the four elements in place of cobalt, as referred to above, showed symptoms of nitrogen deficiency and exhibited retarded growth rates. These effects became increasingly severe as the shoot growth was cut back at five and eight weeks respectively after transplanting seedlings. The appearance of plants with and without cobalt is shown by a photograph (Fig. 1) taken five weeks after the removal of the first axillary shoots. The appearance of the plants that had been supplied with the other metals was similar to that of the cobalt-deficient plants.[16] The deficiency symptoms became very severe, and at the time the photograph was made, 4 out of 16 cobalt deficient plants in the experiment had died. Only one mortality was recorded in cultures receiving other metals in place of cobalt, and no deaths were observed in cultures receiving the cobalt additions. The shoots of plants supplied with cobalt were vigorous in appearance and the foliage was dark green. When the plants were subjected to short day lengths three weeks after the removal of the first axillary shoots, plants in cultures receiving cobalt produced an abundance of flowers, most of which developed into healthy pods. Plants lacking cobalt, on the other hand, produced fewer flowers, and many of these failed to set fruit. Many of the pods that formed on cobalt-deficient plants were shed prematurely.

The data illustrating the yield of dry shoots, roots, nodules, and seed and that showing the viability of seed are presented in Table 1. These results corroborate the visual observations listed above. The effect of cobalt in comparison to no cobalt or to the other metal additions in general is reflected by the production of consistently greater dry weights of shoots, roots, root nodules, and seed. It is apparent

from the data that the dry weights of initial shoots from cultures supplied with cobalt at 0.1 and 1.0 ppb were respectively 1.5 and 1.3 times those of the initial shoots from cultures lacking the element. The dry weights of the first axillary shoots obtained from cultures receiving 0.1 and 1.0 ppb of cobalt were, respectively, 4.5 and 3.8 times those of controls. In the final harvest of the second axillary shoots, the dry weight yields obtained from the 0.1 and 1.0 ppb of cobalt treatments respectively had risen to 14.4 and 12.6 times that of comparable shoots produced by cultures lacking cobalt. This trend of response very likely illustrates a progressive removal of the endogenous cobalt derived from the seed used for the experimental plants.

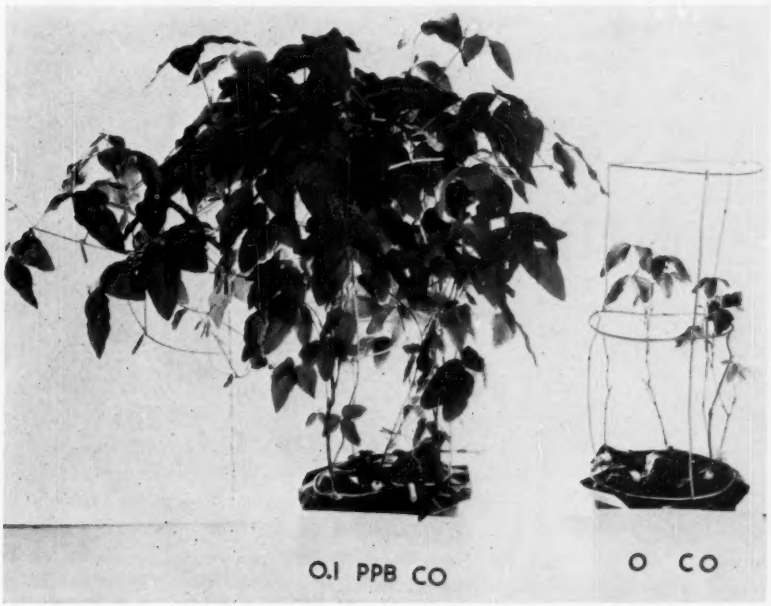

FIG. 1.—An illustration of the effect of 0.1 ppb of cobalt on the growth of the second axillary shoots of soybean plants cultured under symbiotic conditions. The photograph was made five weeks after harvesting the first axillary shoots.

The yield of dry roots was markedly affected by the addition of cobalt to culture solutions. The magnitude of the response, however, was not as great as that of the second axillary shoots, but roots were not subjected to the cutting-back technique that was employed with shoots.

In comparison to the effects of cobalt on the production of dry weight of the different plant parts, the effect of the other metals was not particularly striking. Small increases in growth resulting from addition of these elements appeared to be consistently reflected in an increased production of second axillary shoots, roots, root nodules, and seed. A statistical test of these treatments as a population separate from the population supplied with cobalt showed that excepting for the production of seed (weight), none of the other differences were statistically significant.

The effect of cobalt on the number of viable seed produced is remarkably great and provides strong evidence for the essentiality of the element. The effect of other elements on the production of viable seed in no way approaches the effect of cobalt. A statistical examination of the data for all those treatments lacking cobalt revealed that neither the V nor the Ni-Ga-Al treatment resulted in a significant increase in the number of viable seed. As indicated in Table 1, the differences between the numbers of viable seed produced by the treatment lacking cobalt and those produced with metal additions other than Co were not statistically significant when all the treatments were included in the statistical analysis.

Effects on chemical composition: In order to study the effect of cobalt and other added elements on the composition of the plants, the contents of total nitrogen, chlorophyll, hemoglobin, vitamin B_{12}, and cobalt were determined in certain plant parts. It is obvious from the data in Table 2 that the total nitrogen in the whole

TABLE 2

EFFECT OF COBALT AND OTHER ELEMENTS ON THE TOTAL NITROGEN CONTENT OF SOYBEAN PLANTS GROWN UNDER SYMBIOTIC CONDITIONS

	Mg/culture*					
	Shoots					
Treatments	Initial	1st axillary	2nd axillary	Roots†	Seed	Total
No Co	104	87	61	50	22	324
1 ppb each of Ni, Al, and Ga	94	114	137	65	126	536
1 ppb V	91	98	149	72	82	492
0.1 ppb Co	164	496	1,273	345	1,611	3,889
1.0 ppb Co	140	447	1,343	328	1,702	3,960
Least significant difference at 5%	39	116	304	74	102	635
Least significant difference at 1%	55	163	426	104	144	892

* Means of determinations on four replicate cultures.
† Determinations include both roots and root nodules.

plants that were supplied with cobalt was about 12 times that of the control plants which lacked cobalt. The effect on the total nitrogen content of whole plants of metal additions other than cobalt was relatively small and was not statistically significant. Also, it is apparent from the data that the effect of cobalt on the total nitrogen content of shoots became increasingly pronounced as initial and subsequent axillary shoots were harvested from the cultures. The percentage of nitrogen in the shoots (data not presented) followed similar trends. The effect of

TABLE 3

EFFECT OF COBALT AND OTHER METALS ON THE CHLOROPHYLL CONTENT OF LEAVES AND THE HEMOGLOBIN AND VITAMIN B_{12} CONTENT OF ROOT NODULES FROM SOYBEAN PLANTS GROWN UNDER SYMBIOTIC CONDITIONS

			Vitamin B_{12},† (Mμg/gm)*	
Treatments	Chlorophyll (Mg/gm)*	Hemoglobin (Mg/gm)*	L.L. assay	O.M. assay
No Co	2.11	115	18	0.33
1 ppb each of Ni, Al, and Ga	2.19	142	15	0.35
1 ppb V	2.29	115	18	0.31
0.1 ppb Co	3.50	162	26	0.69
1.0 ppb Co	3.30	178	115	2.77
Least significant difference at 5%	0.41	31	15	0.9
Least significant difference at 1%	0.58	44	21	...

* All units are expressed on a fresh-weight basis and are means of determinations on samples from four replicate cultures. Samples for all determinations were collected at time of harvesting first axillary growth (Table 1).
† Determinations by the *Lactobacillus leichmannii* (L.L.) assay indicate apparent B_{12}, while determinations with *Ochromonas malhamensis* (O.M.) are relatively specific for vitamin B_{12}.

TABLE 4

EFFECT OF COBALT AND OTHER ELEMENTS ON THE COBALT CONTENT OF SOYBEAN PLANTS GROWN UNDER SYMBIOTIC CONDITIONS

Treatments	P.p.m. in dry matter			
	Shoot growth			
	Initial	1st axillary	2nd axillary	Roots
No Co	<0.01*	<0.02	<0.02	0.05
1 ppb each of Ni, Al, and Ga	<0.02	<0.01	<0.02	0.05
1 ppb V	<0.02	0.02	0.02	0.09
0.1 ppb Co	0.05	0.03	0.03	0.11
1.0 ppb Co	0.07	0.04	0.04	0.33

* Cobalt determinations on composite samples (ranging from 9 to 34 gm) falling below the detectable range of 0.25γ by the method used have been reported as less than the figures indicated.

cobalt additions on the total nitrogen in roots was striking, but the magnitude of the response was not as great as that for the second axillary shoots.

At the time of harvesting the first axillary shoots, leaf samples were collected for chlorophyll determinations and root nodules also were collected for hemoglobin and vitamin B_{12} analyses. It is evident from the data presented in Table 3 that cobalt additions resulted in a significant increase in the chlorophyll content of the leaves and in the concentration of the hemoglobin and vitamin B_{12} in root nodules. The other elements added, however, had no measurable effect on these constituents. The true as well as the apparent vitamin B_{12} contents of the nodules as assayed by *Ochromonas malhamensis* and *Lactobacillus leichmannii* respectively were consistently higher in nodules from plants supplied with cobalt than in those from plants deficient in cobalt.

The cobalt content of the various plant parts is presented in Table 4. In general the cobalt content of the roots was higher than that of the shoots, and as would be expected, the concentration in various plant parts increased with increasing supply of cobalt in culture solutions. The cobalt content of shoots from cultures without added cobalt was near or less than the minimum detectable concentration. Roots of plants from cultures lacking cobalt contained slightly above the minimum detectable quantity, and the concentration of the element in roots of plants from cultures supplied with cobalt increased with increasing concentration in the culture solutions.

Alleviation of deficiency symptoms: In order to investigate further the specificity of cobalt for symbiotically grown soybean plants and the capacity of the element to cure the deficiency symptoms, four extra cobalt deficient cultures were utilized for tests after the initial shoot growth had been harvested. When the first axillary shoots had grown for a period of three weeks after harvesting the initial shoots, two of the cultures were supplied with five ppb of cobalt in the culture solution and the other two were left untreated, and, therefore, they served as controls. At the time

TABLE 5

THE EFFECT OF COBALT ON THE SHOOT GROWTH OF SOYBEAN PLANTS SUPPLIED WITH FIXED NITROGEN

Treatments	Dry matter in shoots,* gm./culture	
	Initial	1st axillary
No Co	6.4	17.3
0.1 ppb Co	7.0	19.1
1.0 ppb Co	6.5	15.5
Least significant difference at 5%	Not statistically significant	

Each weight reported represents the mean weight of shoots from four replicate cultures Initial and first axillary shoots were harvested 4 and 7 weeks respectively after transferring seedlings into culture vessels.

of the addition of cobalt, characteristic deficiency symptoms were apparent on the foliage of plants in all four cultures. Within ten days, the cobalt addition resulted in definite evidence of alleviation of the deficiency symptoms. The appearance of the treated and untreated plants five weeks after addition of cobalt to the deficient cultures is shown by a photograph (Fig. 2). Three weeks after adding cobalt, the new leaves of treated plants appeared dark green, and a random sample of the third trifoliate leaves below the growing point of both deficient and treated plants showed that the addition of cobalt had caused a marked increase in both the contents of chlorophyll and total nitrogen. The results of these analyses already have been reported.[16]

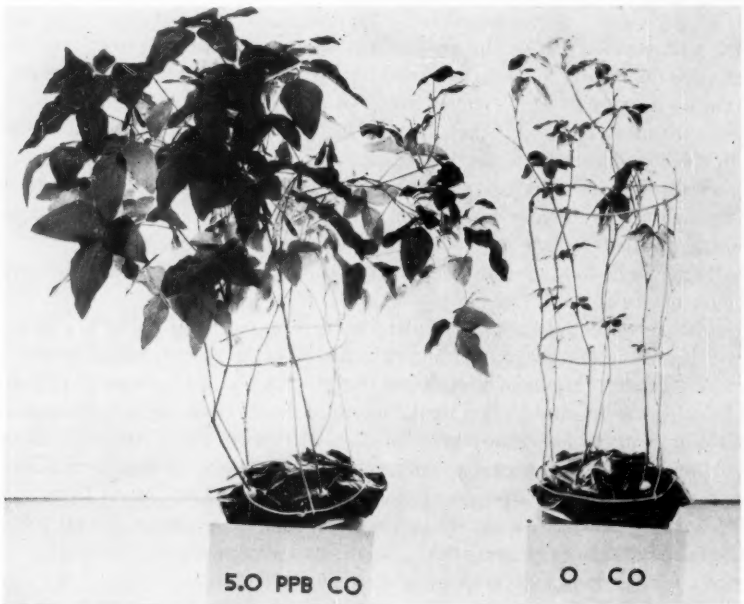

FIG. 2.—An illustration of the capacity of cobalt to cure deficiency symptoms of the first axillary shoot growth of soybean plants cultured under symbiotic conditions. The photograph was made five weeks after the addition of 5 ppb of cobalt to deficient cultures.

In order to further test the specificity of cobalt, eight other spectrographically standardized elements, viz. Ru, Bi, Sn, Cr, U, Ag, W, and Pd, each at a concentration of one ppb, were supplied to one of two control cultures. This mixture was added four weeks after the initial growth was harvested. Although these cultures were maintained for three weeks after the addition, no change in the deficiency symptoms took place. All of the four cultures were harvested seven weeks after harvesting the initial axillary growth, and the dry weights of shoots and roots and the air-dry weights of seed were recorded. The dry weights of shoots, including the dry weight of the pericarps, in grams per culture were 10.7 for plants lacking Co, 6.3 for those receiving one ppb of each of the eight elements, and 27.2 for those

cured with five ppb of cobalt. The corresponding dry weights of roots in grams per culture were 6.1, 7.6, and 8.2 for the three treatments respectively. The air-dry weights of the seed were 2.4, 0.5, and 13.4 respectively for no cobalt, the eight-element addition, and the five-ppb cobalt addition. The analysis of the samples for total nitrogen showed that the shoots from plants receiving no cobalt, the eight-element addition, and the five-ppb cobalt addition contained respectively 1.8, 1.6, 2.5 and per cent total nitrogen.

Although the number of cobalt-deficient cultures availabe for specificity tests was less than desirable, it seems clear from these and other results (Table 1) that cobalt is highly specific in its capacity to cure or to prevent deficiency symptoms.

Effects on Non-Symbiotic Plant Growth.—The results of the experiment presented above clearly show that cobalt is essential for the growth of soybean plants that are inoculated with *Rhizobium japonicum* and forced to depend upon the fixation of atmospheric nitrogen. In view of these results, it was of obvious interest to attempt to determine whether or not soybean plants that were supplied with adequate fixed nitrogen and were devoid of nodules would exhibit any response to this element.

The experiment outlined in Table 5 involved a control treatment lacking cobalt one treatment with cobalt at 0.1 ppb, and another with cobalt at 1.0 ppb. All culture solutions contained adequate fixed nitrogen, and all plants were devoid of root nodules. Initial shoots and the first axillary shoots were harvested from the cultures in this experiment, and as indicated by the data in Table 5, the addition of cobalt failed to produce a statistically significant effect on the yield of shoots. The mean total nitrogen content of the samples (per cent in dry matter) derived from the initial shoot growth was 4.6 for no cobalt, 4.6 for 0.1 ppb of cobalt, and 4.5 for the 1.0 ppb cobalt treatment, and the mean total nitrogen content (per cent) of samples from the first axillary growth was 4.7, 4.8, and 4.9 for the respective treatments. The chlorophyll contents of leaves in milligrams per gram of fresh weight for the various treatments were 2.8 for no cobalt, 2.9 for 0.1 ppb of cobalt, and 2.8 for 1.0 ppb of cobalt. Neither the nitrogen nor the chlorophyll contents of plant material from cultures receiving the different cobalt treatments varied significantly. In this same experiment, second axillary shoots were allowed to grow after the first axillary shoots were harvested, and still no beneficial effects of cobalt were observed. It seems apparent that this experiment did not produce evidence of a cobalt requirement for soybean plants grown with adequate nitrogen. In contrast, plants grown under strictly symbiotic conditions either prior to this experiment or simultaneously with it exhibited severe deficiency symptoms without cobalt in the nutrient solutions.

Discussion.—In a series of experiments[13, 16] including those presented herein, it has been consistently observed that soybean plants cultured under strictly symbiotic conditions and supplied with highly purified nutrient solutions exhibit a definite requirement for traces of cobalt. The effect of cobalt is relatively specific since 12 other elements selected on the basis of their chemical properties and their possibilities of being removed by the purification procedure failed to alleviate deficiency symptoms. The striking effects of cobalt on the growth and composition of soybean plants cultured under symbiotic conditions without combined nitrogen and the lack of any measurable effect of the element on the growth of soybean plants supplied with purified nutrient solutions containing adequate fixed nitrogen suggest

that cobalt plays its role in the metabolism of the *Rhizobium* within the nodules. The pronounced effect of cobalt on the nitrogen content of plants and the apparent nitrogen-deficiency symptoms in the absence of the element suggests a role in the nitrogen-fixing process. It has been established by these experiments and others[10] that nodules of leguminous plants contain vitamin B_{12}. It is known also that pure cultures of nitrogen-fixing bacteria excrete vitamin B_{12} into culture media.[8] No information is available, however, on the cobalt requirements for growth of *Rhizobia* in purified media. Information on this point obviously is essential for a satisfactory understanding of the role of cobalt in symbiotically grown soybean plants. In view of the strong evidence that the synthesis of vitamin B_{12} is limited to microorganisms, it seems probable that a major role of cobalt in symbiotically grown soybean plants will prove to be connected with the metabolism of the *Rhizobium japonicum* cells within nodules. An association of cobalt with the nitrogen-fixing process was suggested by studies on the cobalt requirements of blue-green algae by Holm-Hansen *et al.*[9] The conclusion of these workers is in general agreement with our findings that the necessity of cobalt for soybean plants was limited to those conditions where symbiotic nitrogen fixation was obligatory. It is obvious, of course, that cobalt may be shown to fulfill functions in living organisms other than its function as a constituent of vitamin B_{12}.[28, 29]

It seems apparent that the concentration of vitamin B_{12} or apparent vitamin B_{12} in soybean nodules is positively correlated with the cobalt supply of culture solutions but the relationship of this vitamin or of a coenzyme containing it to the metabolism of bacteria within the nodule environment remains for future investigation. The apparent B_{12} content of nodules as measured by *Lactobacillus leichmannii* was consistently greater than the B_{12} as measured by *Ochromonas malhamensis*. This difference in the two assays was expected, since it has been reported[30] that 90 per cent of the vitamin B_{12} activity measured with *Lactobacillus leichmannii* could be due to compounds other than true vitamin B_{12}.

The results of these investigations do not support the view that cobalt plays an essential role in the metabolism of the soybean plant *per se*. This conclusion is in contrast to the findings of Bolle-Jones and Mallikarjuneswara,[12] who demonstrated beneficial effects on the growth of both rubber and tomato plants. The results presented in this paper, however, do not rule out the possibility of a cobalt requirement for nonsymbiotically grown plants. If future experimentation establishes a cobalt requirement for higher plants supplied with adequate nitrogen, it seems highly probable that the magnitude of the requirement will be considerably less than that established for symbiotically-grown soybean plants.

Summary.—Experiments have been conducted to determine the effect of cobalt on the growth and composition, in respect to certain constituents, of soybean plants provided with highly purified nutrient solutions. The addition of cobalt at 0.1 or 1.0 ppb to nitrogen-free nutrient solutions supplied to soybean plants cultured under symbiotic conditions resulted in marked increases in both total weight and nitrogen content of the plants. The magnitude of the response increased as initial shoots were removed and subsequent axillary shoots were harvested from the plants. Cobalt at a concentration of 1 ppb resulted in a 12-fold increase in the dry weight and a more than 22-fold increase in the total nitrogen content of the second axillary shoots. Also, the addition of cobalt to cultures resulted in a striking increase in

the chlorophyll content of leaves and the vitamin B_{12} content of nodules. The effect on the concentration of hemoglobin in nodules was statistically significant but was less striking than the effect on other components. Of the control plants grown under symbiotic conditions without added cobalt, 25 per cent died and those surviving produced a mean of only eight viable seed per culture of four plants. In contrast, no plants died in cultures supplied with 0.1 or 1.0 ppb of cobalt, and these produced means respectively of 256 and 237 viable seed per culture. Twelve elements not considered as essential for higher plants were added to cultures either singly or as mixtures in order to test the specificity of cobalt as an essential cation. None of the additions tested would substitute for cobalt as a growth factor for the symbiotically-grown plants.

In another experiment, the effects of cobalt on the growth of soybean plants were studied by utilizing purified culture solutions containing adequate fixed nitrogen. No evidence of a cobalt requirement was obtained under these conditions.

It is concluded that cobalt is an essential element for the growth of soybean plants under the conditions that force them to depend upon the symbiotic relationship with *Rhizobium japonicum* for the fixation of atmospheric nitrogen.

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† These materials were obtained from Johnson Matthey and Co., Ltd., London.

‡ In this paper the abbreviation "ppb" refers to parts in 10^9 parts and is the American rather than the English usage.

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AUXIN-KINETIN INTERACTION REGULATING THE SCOPOLETIN AND SCOPOLIN LEVELS IN TOBACCO TISSUE CULTURES

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Tobacco tissue cultured *in vitro* has been observed to release a strongly ultraviolet fluorescent material into the agar medium. The intensity of the fluorescence varies with the composition of the medium and the age and other conditions of the cultures. It is especially striking in the presence of high concentrations of 3-indoleacetic acid (IAA). A major fraction of the fluorescent material has been isolated and identified as scopoletin (6-methoxy-7-hydroxycourmarin; 6-methoxy-7-hydroxy-1,2-benzopyrone). Influences of auxins and kinetin on its formation, its liberation from a glycosidic bound form, and its release into the medium will be reported.

Materials and Methods.—Cultures of tobacco tissue (pith sections, stem segments, and especially subcultures of callus), Nicotiana tabacum, variety Wis. #38 were grown in 125-ml Erlenmeyer flasks with 50 ml. of modified White's nutrient agar medium as described by Miller and Skoog[1] or Jablonski and Skoog[2] for periods of 1 to 60 days in different experiments and with various growth substances added to the culture medium. The cultures were kept behind glass doors in cabinets at a room temperature of about 25°C and in the presence of continuous, weak, diffuse light from fluorescent daylight tubes in ceiling fixtures.

Extraction of Scopoletin.—a. *Free scopoletin in the medium:* The fluorescent material was extracted from the medium with freshly redistilled chloroform. Best's[3] procedure for the extraction of scopoletin from tobacco tissues was employed. All operations were carried out in dim light to minimize photoinactivation of the fluorescent substance.

In the preparation of samples large enough for investigations of chemical properties, six liters of agar medium (from 150 flasks) were melted in an Arnold steamer, pooled, and made up to seven liters with distilled H_2O. The pH was adjusted to 2.0 with 1 *N* HCl. The material was allowed to cool to room temperature for one hour in a dark room and was then extracted with three 1-liter portions of chloroform. The chloroform extracts were pooled and the fluorescent material taken up in dilute KOH solution (2 successive 200-ml portions of 0.005 *N* KOH). These alkaline portions were combined, acidified to pH 3 with HCl, and extracted with two 200-ml volumes of chloroform. The chloroform layer was freed from traces of water with anhydrous neutral $CaCl_2$ powder and passed through a 2- × 30-cm alumina column (Bio-Rad Laboratories, 100–200 mesh, activity grade 1). The fluorescent material was retained in the upper one cm of the column. The column was washed with *ca.* 50 ml of chloroform and then with *ca.* 50 ml 9:1 v/v solvent

mixture of petroleum ether and butanol. The latter was used to separate out two bands with a weak blue and yellow fluorescence respectively. The upper one cm of the column was removed and its remaining fluorescent material was eluted with 0.005 *N* KOH. The KOH filtrate was acidified to pH 3, extracted with chloroform which was then dried with neutral $CaCl_2$, filtered, and evaporated to dryness under vacuum at room temperature. A residue of *ca.* five mg total dry weight was obtained.

For quantitative comparisons of amounts of substance released into the medium, the agar from 10 flasks (about 500 cc) was pooled, made up to 600 ml, and adjusted to pH 2. In these cases, one ml 0.005 *N* H_2SO_4 was added to each flask before the agar was melted in the Arnold steamer. Duplicate 100-ml samples of the liquefied agar were transferred to separatory funnels and extracted with four successive 100-ml. volumes of chloroform. The pooled chloroform extract was dried with neutral $CaCl_2$, filtered and chromatographed on a neutral alumina column (0.7 × 20 cm). The column was washed first with chloroform and then with a 9:1 v/v petroleum ether–butanol mixture. The top one cm of the column was removed and eluted with successive portions of pH-10 borate buffer to make 100 ml of eluate. The concentration of scopoletin in 20 ml aliquots was determined from the intensity of the fluorescence in a Coleman photofluorimeter, Model 12, with filters B_1 (365 mμ) and PC-1. The values obtained were in the range from 2×10^{-3} to 1×10^{-2} μg/ml as determined by interpolation on a standard linear concentration curve obtained with a sample of pure chemical† dissolved in pH-10 buffer.

b. *Free scopoletin in tissues:* For determinations of free scopoletin in tissues, samples of approximately one gm fresh weight were dropped into boiling, pH-2 citrate-phosphate buffer (*ca.* 20 ml) for 2 min, ground in a Potter-Elvehjem homogenizer, and centrifuged. The supernatant was decanted and the residue washed with successive 30-ml portions of buffer until all fluorescent material was removed (usually 3 times). The buffer solutions were pooled and made up to 150 ml, and the scopoletin was determined quantitatively as above. The bound scopoletin present in the tissue was retained in the aqueous supernatant from which free scopoletin had been extracted with chloroform.

c. *Extraction from the tissue, hydrolysis, and estimation of the bound form of scopoletin:* In analysis of tissue samples for bound scopoletin, ordinarily the aqueous supernatant remaining after chloroform extraction of free scopoletin, as above, was acidified with 6 *N* HCl (1 volume to 5 of the tissue extract) and refluxed for one hour. The solution was then adjusted to pH 2 with NaOH, and the liberated scopoletin was extracted and estimated as above.

To prepare a sample of the bound form, 300 gm fresh weight of tobacco callus tissue which had been cultured for 40 days on modified White's basal medium with 2.0 mg/l IAA and 0.20 mg/l kinetin were plunged into 300 ml of hot ethanol, kept boiling for 3 min, and homogenized in a Waring blender. The homogenate with Cellite added was filtered through a Gooch filter. The filtrate was concentrated to 10 ml under vacuum at 30°C. A precipitate formed in this process was filtered off, and the concentrate was chromatographed, deposited as a line on a 40- × 40- × 0.3-cm sheet of Eaton Dikeman #320 filter paper, and developed with distilled water. The fluorescent band at approximately R_f 0.80 was eluted with 80 per cent ethanol, concentrated to 2 ml, and rechromatographed with *n*-butanol:acetic acid:water (4:1:2 v/v) as developer on a sheet of Whatman #42 filter paper. The fluorescent material (R_f 0.50) was eluted with 50 per cent ethanol, evaporated to dryness under vacuum, and its properties investigated.

Ultraviolet adsorption spectra were measured with a Beckman Model D. U. spectrophotometer. Fluorescence was measured in accordance with Goodwin and Kavanagh[4] except that as above a Coleman photofluorimeter was employed.

Characterization of the Fluorescent Substance in the Medium.—About five mg of the fluorescent material was extracted from agar medium on which tobacco callus tissue had been grown and was obtained in relatively pure state by chromatography procedures as described above.

Spectral and chromatographic properties: As may be seen from Figure 1 (upper curve), the absorption spectrum of the extracted material closely matches that of a standard solution of scopoletin throughout the tested range (from 220 to 360 mμ) with maxima at 229, 255, 298, and 343 mμ and with minima at 250, 269, and 308 mμ.

Such minor differences as occur may be readily accounted for by concentration effects and by impurities present in the extract.

The fluorescence of the extracted material in ultraviolet light has a maximum intensity above pH 8 and is relatively weak below pH 6. The change in intensity with pH is very nearly the same as for the standard scopoletin sample, as is shown by the curve in Figure 2, and matches Goodwin's and Kavanagh's previously published curves for scopoletin.[4]

Chromatographic properties of the material extracted from the medium also have been compared with those of the scopoletin sample as follows: The two substances were co-chromatographed as separate spots and as combined single spots on Whatman #1 paper with various solvent systems as indicated in Table 1. In all cases,

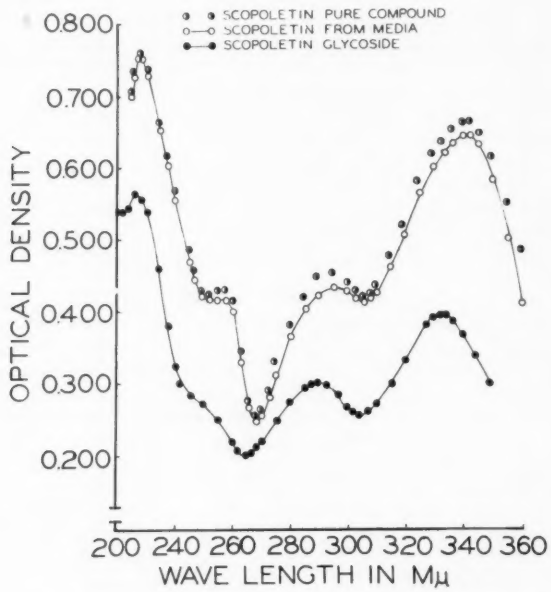

FIG. 1.—Absorption spectra of scopoletin (5×10^{-6} *M*) and of fluorescent materials from tobacco callus tissue cultures.

the substances run together as a single compound with the R_f values listed in the column headed scopoletin. It is concluded, therefore, that the fluorescent substance extracted from the medium is in fact scopoletin.

Free scopoletin in the tissue: The callus tissue itself tends to contain relatively low concentrations of free scopoletin. Typical amounts are represented by the curves in Figs. 4, 5, and 6, i. e., on the order of 5 μg/gm. of fresh growing tissue.

Characterization of a Bound Form of Scopoletin in the Tissue.—Most of the scopoletin in the tissue is present in a bound form which has been estimated in terms of the scopoletin set free by acid hydrolysis. This bound form apparently is retained entirely within the cells; at least, it could not be detected in the medium. The

properties of one sample of the material extracted from 300 gm. fresh weight of callus and prepared as described above have been investigated.

As shown in Figure 1, the UV absorption spectrum of this bound form (lower curve) resembles that of free scopoletin but shows slight shifts in the positions of maxima from 229, 298, and 343 to 225, 290, and 334 mμ respectively and of minima from 260 and 308 to 265 and 305 mμ respectively. A possible difference between the curves exists in the 250–260 mμ region where the bound form for some reason gives at most a hint of the shelf exhibited by the free scopoletin.

The fluorescence intensity–pH curve of the bound form, as shown in Figure 2, is strikingly different from that of free scopoletin in being much higher in strong acid, in being practically constant between pH 1 and 11, and in falling rapidly around pH 12. This curve is similar if not identical to that of the scopoletin glycoside reported by Goodwin and Pollock[5] (Fig. 3, p. 518 of ref. 5) to be present in *Avena* roots.

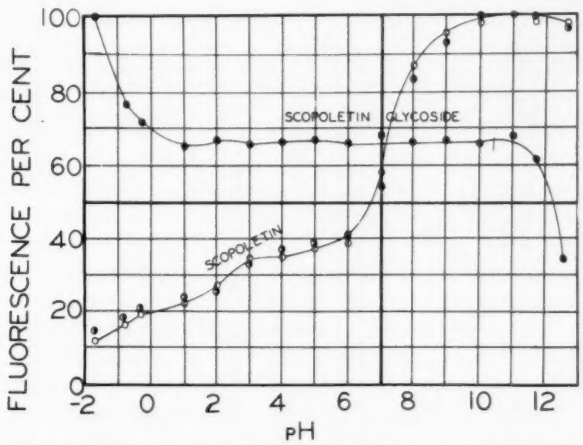

FIG. 2.—Effect of pH on the intensity of fluorescence of a standard scopoletin sample (lower curve), of material (scopoletin) leached by tobacco callus tissue into the medium (half-filled circles), and of a bound form of scopoletin (scopolin) obtained in water extracts of the tissue (upper curve).

Also the R_f values obtained in paper chromatograms with different solvent mixtures are quite different for scopoletin and this substance, as may be seen by comparing columns 1 and 2 in Table 1.

Evidence that the fluorescent material in the bound form actually is scopoletin was obtained by co-chromatographing its hydrolysate with the pure substance. As shown in Figure 3, the hydrolysate (H) gives a fluorescent compound with the identical Rf value as scopoletin. Also the pH-fluorescence curve of the eluate of this spot is identical with that of scopoletin. Two additional spots are obtained in chromatograms of the hydrolysate by spraying with alkaline 3,5-dinitrosalicylate—spots not obtained in chromatograms of the untreated material. In co-chromatograms with sugars (Fig. 3), these spots correspond to those of glucose and fructose with Rf values 0.25 and 0.31 respectively in butanol : acetic acid : water (4:1:2). These results strongly suggest that the substance is a glycoside and that sucrose

TABLE 1

R_F VALUES OF SCOPOLETIN AND ITS ABUNDANT GLYCOSIDE, OBTAINED FROM TOBACCO CALLUS TISSUE, FOR VARIOUS SOLVENT MIXTURES AT *CA.* 28°C

Solvent mixture developer	R_F Scopoletin	R_F Scopoletin glycoside
Water	0.45	0.85
Acetic acid 10%	0.57	0.84
n-Butanol:acetic acid:water (4:1:2)	0.85	0.50
n-Butanol:ammonia:water (4:1:5)	0.45	0.55
n-Butanol:water (4:1)	0.85	0.40
Ethyl acetate:2 *N* HCl (1:1)	0.95	0.10
Amyl alcohol:acetic acid:water (4:1:5)	0.88	0.30
Isopropanol:ammonia:water (10:1:1)	0.75	0.80

might be the glucone. It is of interest to note the close agreement between these findings and the results reported by Goodwin and Kavanagh[6] for one of the glycosides they isolated from *Avena* roots. However, the possibility that the composition

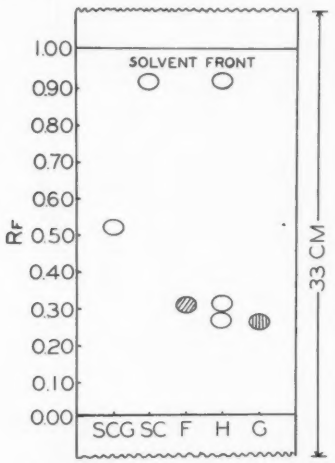

FIG. 3.—Chromatogram of scopoletin (SC), scopoletin glycoside (SCG), SCG-hydrolysate (H), fructose (F), and glucose (G). The developer was *n*-butanol:acetic acid:water (4:2:1 v/v) on Whatman No. 1 filter paper.

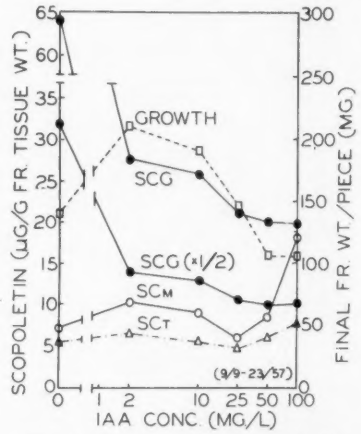

FIG. 4.—Effects of IAA concentration on growth of tobacco callus, on the levels of free and bound scopoletin in the tissue, and on the content of scopoletin leached into the medium. SC_T, scopoletin in tissue; SC_M, scopoletin in medium; SCG (× 1/2), scopoletin glycoside in tissue (half scale); growth, average final fresh weight of callus. Cultures harvested after 14 days growth. Kinetin concentration 0.2 mg/l.

may involve fructose and glucose separately, or even some other sugars, is of course not excluded.‡

Factors Influencing the Appearance of Scopoletin in the Medium.—Effects of auxins: As mentioned above, this investigation started from the observation that in the

presence of IAA a strong, bluish U.V. fluorescence would appear in the agar medium on which tobacco tissue was grown. With relatively high IAA concentrations (0.10 mg/l or more) this fluorescence became noticeable within four hours after the cultures had been started, even though only three pieces of tissue with a total volume of *ca.* 0.15 ml were placed on the surface of a 50-ml volume of agar. With lower IAA concentrations, longer times were required for the fluorescence to appear clearly, but even in control cultures without added IAA, it was definitely visible after two days. The rate of release of scopoletin has not been studied quantitatively, but the influence of the added IAA concentration on the amounts of scopoletin present in the medium of 1- to 4-week-old cultures has been determined in a series of 12 experiments performed within a fifteen-month period. Quantitative determinations based on measurements of the fluorescent material in extracts of the medium and of tissues in relation to the growth of the tissues have been made in five experiments with serial concentrations of IAA. Results of one typical experiment are presented in Figure 4. It may be seen that the amount of scopoletin released into the medium (curve SC_M) first increases with the concentration of IAA and then falls gradually, more or less in parallel with the effectiveness of the IAA concentration in promoting growth, until a toxic level of IAA is reached. From this point on, there is a marked rise in scopoletin content in the medium, which is correlated with a decrease in final weight of the tissue pieces, especially in the presence of the higher IAA concentrations. Very similar results have been obtained with α-naphthaleneacetic acid (NAA) substituted for IAA (Fig. 5A). With 2,4-dichlorophenoxyacetic acid (2,4-D), the rise and gradual fall of the scopoletin curve in the low dosage range have not been found, but, as shown in Figure 5B, the release of scopoletin by the high concentration is striking. It appears, therefore, that the release of scopoletin into the medium is a general effect of high auxin levels on the tobacco tissue.

The levels of free scopoletin in the tissue (curves SC_T, Figs. 4, 5A, and 5B), are relatively low in healthy tissue (about 5 μg/gm) and increase with the IAA concentration or decrease with the kinetin concentration (Fig. 6), as reflected also in each case by changes in the amounts of scopoletin in the medium (curves SC_M).

The level of scopoletin glycoside in the tissue is also greatly affected by IAA treatments. As shown in Figure 4 by curve SCG (or SCG ($\times^1/_2$), plotted on half the scale of the others), in the presence of low, growth-promoting concentrations of IAA, the glycoside level drops sharply to about a third or fourth the value for controls and then continues to fall gradually with higher IAA concentrations, which give marked increases in the level of free scopoletin in the medium. In the cited experiment, the sharp drop in glycoside level is from 65 to 25 μg/gm. scopoletin equivalents, and in repeated experiments, drops from the 80–60 μg/gm range to the 30–20 μg range have been found consistently.

Although auxin treatments enhance breakdown of the bound form of scopoletin in the tissue and the appearance of its free form in the medium, the causal relationship between the two processes is far from a simple one. In the low IAA concentration range, more glycoside is broken down than is accounted for by the sum of free scopoletin present in the tissue and leached into the medium. In higher IAA concentration range, on the other hand, the free scopoletin level in the medium goes up several fold without much change in the content of the bound form in the tissue. Limitations in analytic procedures may be partly responsible for this. In view of

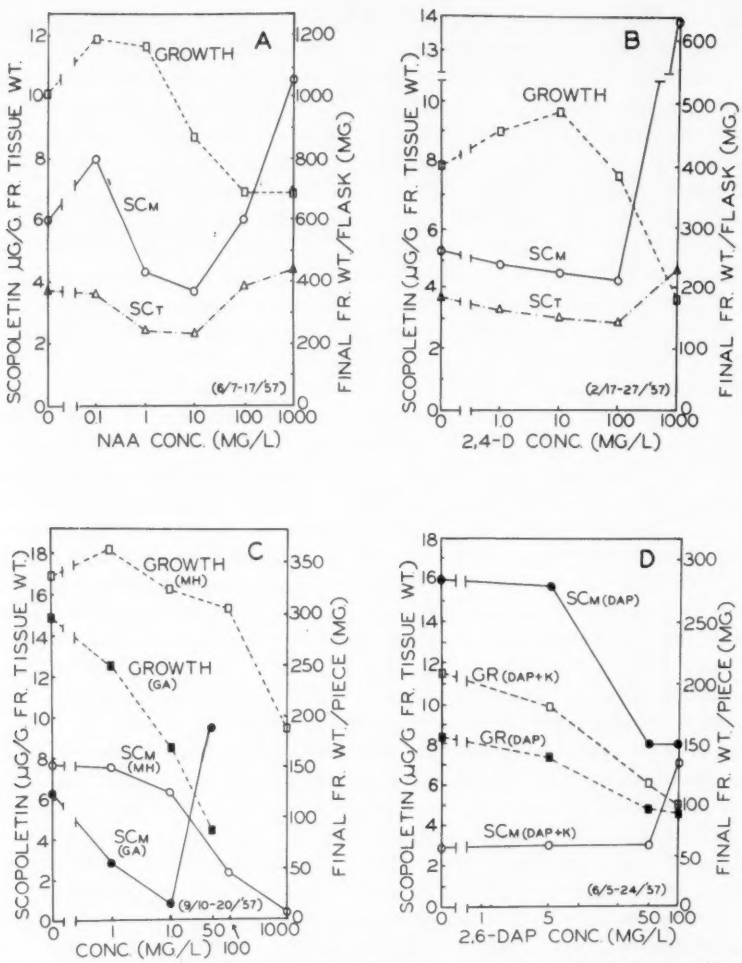

FIG. 5.—Effects of concentration of various substances on growth of tobacco callus and on the content of scopoletin released into the medium. Note that in Figs. 5A and B growth refers to average final fresh weight per *piece* but in Figs. 5C and D to the fresh weight per *flask*. Symbols as in Fig. 4.

A. Treatments with naphthaleneacetic acid (NAA) for a 10-day growth period.

B. Treatments with 2,4-dichlorophenoxyacetic acid (2,4-D) for a 10-day growth period.

C. Treatments with maleic hydrazide (MH) or with gibberellic acid (GA) for a 10-day growth period.

D. Treatments with 2,6-diaminopurine with and without 0.50 mg/l of kinetin (DAP and DAP + K respectively) for a 19-day growth period. IAA, 2.0 mg/l, added in all treatments.

the relative abundance of glycoside, marked shifts in free scopoletin levels might well occur without perceptibly changing the level of the bound form. Only one auxin, IAA, has been tested for an effect specifically on glycoside breakdown, but the

similar action of other auxins in releasing scopoletin into the medium suggests that they all are effective in the same manner as IAA.

Effects of kinetin: In view of the striking interaction of kinetin and IAA in the growth and differentiation of tobacco cultures (see Skoog and Miller[8]), effects of kinetin on the scopoletin status of the tissue was next studied. Results of an experiment in which the kinetin level was varied and that of IAA kept constant at 2.0 mg/l are presented in Figure 6. It may be seen that the cultures with IAA and without kinetin, as expected, show a high free-scopoletin level in the medium (18 μg/gm of tissue) and a low tissue-glycoside level (25 μg/gm as compared with 73 μg/gm of tissue in controls without IAA). With increasing kinetin concentrations, growth increases

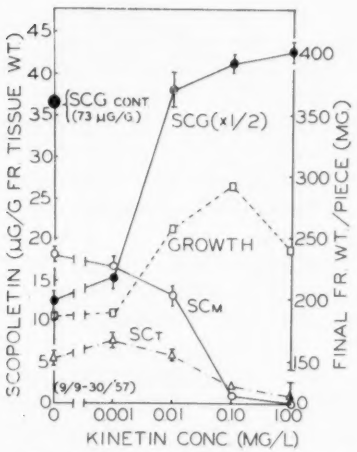

FIG. 6.—Effects of kinetin concentration on the growth of tobacco callus, on the levels of free and bound scopoletin in the tissue, and on the content of scopoletin released into the medium. Symbols as in Fig. 4. Symbols with bars represent means of duplicate determinations differing by the length of the bars. IAA 2.0 mg/l present in all treatments except SCG control. Cultures harvested after a 21-day growth period.

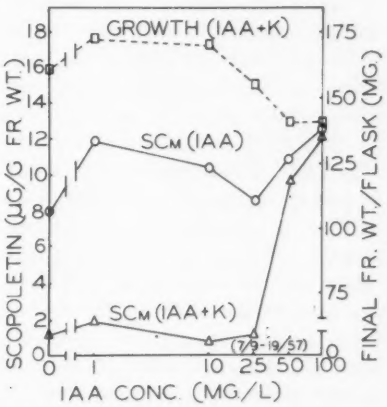

FIG. 7.—Auxin-kinetin interaction in growth of callus and in the release of scopoletin into the medium. Symbols same as in Figs. 4 and 6. Kinetin concentration 0.5 mg/l. Cultures harvested after a 10-day growth period.

to a maximum fresh-weight yield at 0.10 mg/l. Associated with this are a marked lowering of the free scopoletin level in the tissue, a drastic drop practically to zero in the scopoletin content of the medium, and a spectacular rise in scopoletin glycoside in the tissue. Even the relatively minute kinetin concentrations (0.001 and 0.010 mg/l) are effective in raising the glycoside content, and the higher concentrations apparently raise it above the level found in the control without added IAA. In Figure 6, the low kinetin concentrations had only a slight effect in preventing the release of scopoletin into the medium, but in another experiment, 0.001 and 0.010 mg/l kinetin caused decreases of 50 per cent and 90 per cent respectively. Although the absolute values for each determination varied from one experiment to the next, all essential results of this experiment have been confirmed repeatedly.

The maintenance of a high scopoletin glycoside level in the tissue represents a new, remarkably potent biological activity of kinetin, which is strikingly correlated with its activity in promoting growth and differentiation of the callus and which is not exerted in killed or physiologically damaged tissue.

The capacity of a moderate kinetin level (0.5 mg/l) to prevent increasing concentrations of IAA from causing scopoletin to be released into the medium is illustrated by the results shown in Figure 7. Note that in the presence of kinetin, concentrations of IAA up to 10 mg/l increase the yield of tissue but that higher concentrations tend to inhibit growth. In fact, the 50- and 100-mg/l concentrations are highly toxic to the tissue. The free scopoletin in the medium in the absence of kinetin (curve SC_M (IAA)) changes more or less in accordance with earlier results. The effect of kinetin in lowering the scopoletin content in the medium (curve SC_M (IAA + K)) is very striking. It should be noted especially that as little as 0.5 mg/l of kinetin counteracts completely the effects of IAA concentrations up to 25 mg/l but fails entirely to act against the toxic 50- and 100-mg/l IAA concentrations. In other experiments, higher kinetin concentrations, up to 5 mg/l , were used. These were equally ineffective in preventing the release of scopoletin in the presence of toxic IAA levels. This failure may well be due to a generally toxic action of the high IAA levels which perhaps is totally unrelated to the action of this auxin either in promoting growth or in splitting scopoletin glycosides.

That the effect of kinetin in preventing leaching of scopoletin is not merely a consequence of its growth-promoting action is suggested by results of an experiment in which cultures with 2.0 mg/l IAA and 0.5 mg/l kinetin were treated with increasing concentrations of 2,6-diaminopurine (DAP). This substance tends to stop both the division and the enlargement of cells. As shown in Figure 5D, the presence of kinetin markedly reduces the scopoletin content in the medium even when growth of the tissue is almost completely inhibited by the DAP. Results obtained with the highly toxic (100 mg/l) DAP concentration are of course questionable and may be disregarded.

It is of interest that extracts of plant materials which give kinetin-like effects on growth of callus tissue (i. e., possess kinin activity) also exert effects similar to that of kinetin in lowering the scopoletin content in the culture medium. Effects of coconut milk and of a copra extract are shown in Table 2. A comparable effect has been obtained with a beet leaf concentrate with kinin activity. Several other substances with different biological activities which have been tested, if they were effective at all, have tended to increase the scopoletin content in the medium. The

TABLE 2

EFFECTS OF COCONUT MILK OR COPRA MEAL EXTRACT ON THE GROWTH OF TOBACCO CALLUS AND ON THE CONTENT OF SCOPOLETIN RELEASED INTO THE NUTRIENT AGAR

Experiment	Treatments: Material	Concentration,* %	Average final dry weight mg/flask	Scopoletin in medium, μg/gm dry tissue
I (30 days: 11/19/56 to 12/19/56)	Coconut milk	0	49	93
		15	89	19
		45	44	15
II (26 days: 12/15/56 to 1/10/57)	Copra meal extract	0	61	83
		15	100	16.5
		30	55	17

* Concentration of coconut milk is volume per cent. The bottom two figures in the concentration column refer to extracts obtained from amounts of copra meal equivalent to 15 and 30 per cent respectively by weight of medium.

TABLE 3

EFFECT OF PHENYLALANINE ON THE RELEASE OF SCOPOLETIN FROM TOBACCO CALLUS INTO THE CULTURE MEDIUM

Experiment	Treatments, mg/l IAA	Phenylalanine	Average final dry weight mg/flask	Scopoletin in medium μg/gm fresh tissue
I (61 days: 12/14/56 to 2/13/57)	0	0	121	40
	0	50	73	91
	0	100	26	208
	0	150	18	300
	0	300	18	833
II (56 days: 6/11/57 to 8/6/57)	2	0	176	82
	2	50	103	161
	2	100	51	230
	2	150	23	310
	2	300	23	880

effects of gibberellic acid and of maleic hydrazide are shown in Figure 5C. It is of interest that the effect of gibberellic acid in this as in several other physiological respects in part resembles, but still is clearly distinct from, an effect of auxins.

In preliminary experiments, phenylalanine, generally considered to be a precursor of phenolic compounds, seemed to be highly active in releasing scopoletin into the medium (Table 3). The apparent failure of tyrosine, which also serves as a source of phenolic substances, to influence the scopoletin content in the medium in a similar experiment is perhaps misleading.

Effects of Scopoletin on Growth of Tobacco Tissues.—One interpretation of the observed interrelationships between effects of IAA and kinetin on scopoletin levels and on growth might be that auxin serves to release scopoletin, which then in turn reacts with kinetin to promote growth. Both promoting and inhibiting effects of scopoletin on growth have been reported by Andreae *et al.*[10] and Goodwin *et al.*[12, 13] To test this simple notion, tobacco callus and excised pith tissues were cultured on media with and without kinetin and with serial concentrations of either scopoletin or IAA. As shown in Figure 8, the lowest concentration (5 mg/l) of scopoletin appears to be about equally effective as IAA in promoting growth (increase in fresh weight) of the callus in a ten-day period. The action of the two substances differs in that scopoletin, regardless of the concentration employed, is unable to sustain growth over long periods or in successive transfers to new medium. Cytologic examination of sections and squash preparations of the callus by Dr. N. K. Das has shown that the effect of scopoletin like that of IAA was mainly on enlargement of the cells. In fact, the action of scopoletin in this case is the same as would be expected from a low IAA concentration in combination with the employed level of kinetin (see Fig. 9). That scopoletin does not act merely as a weak auxin, however, is clear from the results

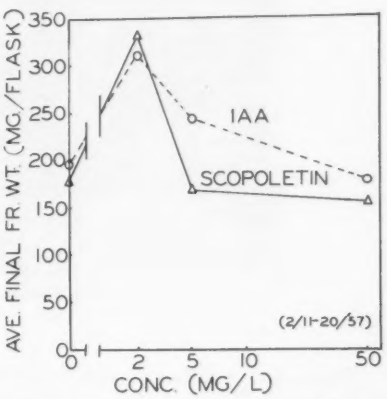

FIG. 8.—Effects of concentration of scopoletin and of IAA on the growth of tobacco callus. Average initial fresh weights *ca.* 150 mg/flask. Growth period, 9 days.

obtained with pith tissue, illustrated in Figure 10. The pith tissue, in contrast with callus, does not produce sufficient auxin to grow without an exogenous auxin supply, and in this case, scopoletin has no visible effect on growth either in the absence or presence of kinetin. These results permit the conclusion that scopoletin only promotes the growth of tissue the auxin content of which is above the required limit for growth. Furthermore, as scopoletin did promote the growth of callus but not of pith tissue its action in the former case would not be due to auxin that might be present as an impurity in the sample. The results rather are in agreement with Andreae's[9] finding that scopoletin inhibits indoleacetic acid oxidase and with the suggestion by Andreae and Andreae[10] that its growth-promoting action (when it occurs) is due to its sparing action on endogenously produced auxin.

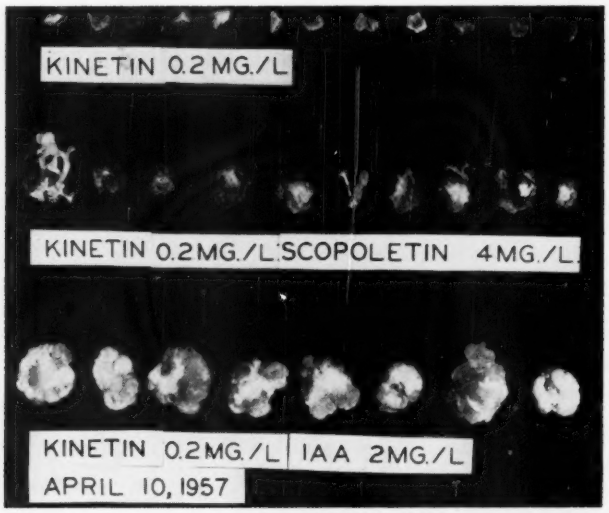

Fig. 9.—Effects of treatments with kinetin, kinetin and scopoletin or kinetin and IAA on size and form of tobacco callus after a 45-day growth period.

Discussion.—Although the exact manner in which auxin and kinetin interact to regulate the scopoletin-scopoletin glycoside levels in the tissue still remains to be determined, work to be presented elsewhere will show it to be closely correlated with these substances in regulating growth of the tissue (Sargent and Skoog[7]) and to involve a highly quantitative coordination of the metabolism of scopoletin and its family of glycosides and perhaps other bound forms (Sargent and Skoog[11]).

Many earlier observations also have pointed to relationships between growth, auxin action, and the contents or metabolism of phenolic substances. Scopoletin was reported by Andreae to stimulate or inhibit the growth of cress and pea roots as as a function of its concentration, and it has been reported by Goodwin and Taves[12] to be a naturally occurring inhibitor of root growth. It also modifies the pattern of differentiation (Avers and Goodwin[13]). Andreae and Andreae first showed that scopoletin acts as a competitive inhibitor of IAA oxidase. On the other hand, an influence of "auxin," 2,4-D , on the scopoletin content of tissues has been reported by

Fults and Johnson.[14] They showed that tobacco plants sprayed with 2,4-D "accumulate" scopoletin and considered this as a factor in the lethal action of the herbicide. Best[15] has shown, furthermore, that following "topping" of tobacco plants, scopoletin accumulates in the root system. It is of interest that bacterial or virus infections which may raise the free auxin levels in plants also appear to cause "accumulation" of scopoletin.

In work with tobacco pith cultures in this laboratory, one of the striking early changes in composition associated with IAA induction of cell enlargement was found to be an increase in the chlorogenic acid content (Wells[16]), and Tryon[17] found the capacities of strains of tobacco callus cultures to form buds to be strinkingly correlated with their scopoletin content.

The present results strongly suggest that increases in free scopoletin which have

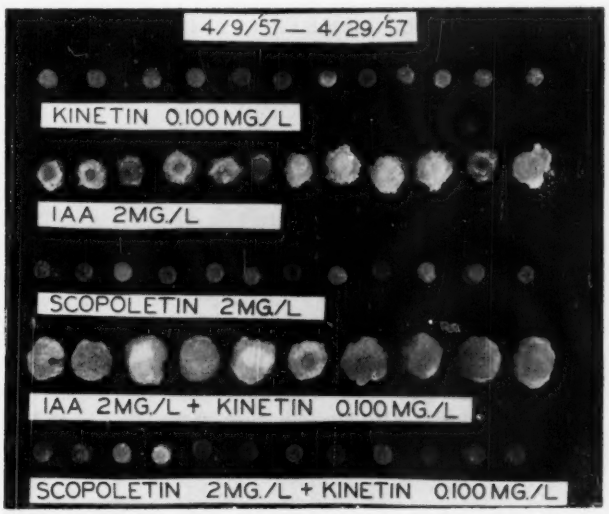

FIG. 10.—Effects of treatments with kinetin, scopoletin, IAA, or pairs of these substances on size and form of tobacco pith disks after a 20-day growth period.

been reported to occur following auxin treatments are brought about through an effect of IAA on the breakdown of bound scopoletin already present in the tissues. It should be noted, however, that a large glycoside loss in the tissues is achieved already with the lowest growth-promoting concentration of IAA, only a small portion of which is accounted for by free scopoletin. Presumably, the glycoside is converted to various cell-wall materials which are increased following treatments with growth-promoting levels of IAA and kinetin. Although exact relationships of such a conversion with reference to growth would not be revealed by the present long-term measurements, the early rise and gradual decrease in free scopoletin associated with increasing, growth-promoting concentrations of IAA would be in agreement with this. The final rapid rise in free scopoletin and the failure of kinetin to counteract it would be logically expected in the presence of high, toxic levels of IAA which stop cell-wall formation and other normal cell functions.

It would appear that relative auxin-kinetin levels regulate the relative rates of formation of different cell-wall components, which in turn may determine the size, form, and organization of cells. The nature of this regulation and its significance in growth and differentiation of the tissue cultures will be further investigated.

Summary.—Tobacco tissue cultured *in vitro* releases into the medium a fluorescent material the main portion of which has been identified as scopoletin. The amount of scopoletin released varies with the concentration of auxin in the medium and increases markedly with high, toxic levels of auxin.

It has been shown that the scopoletin derives from a glycoside (scopolin).

Kinetin added to the medium prevents the release of scopoletin by indoleacetic acid (IAA) and permits the maintenance of high glucoside levels even in the presence of high nontoxic levels of IAA. With toxic IAA levels, treatment with kinetin becomes ineffective. It is suggested that the auxin-kinetin levels regulate the scopoletin–scopoletin glycoside equilibria in the tissues and the conversion of these substances into cell-wall materials. This process is considered as a part of the system in which auxin and kinetin interact in regulating growth and organ formation in plant tissues.

This work was supported in part by the Research Committee of the Graduate School, University of Wisconsin, with funds from the Wisconsin Alumni Research Foundation and by grant G5390 to F. Skoog from the National Science Foundation. E. Montaldi is grateful to the John Simon Guggenheim Foundation for a fellowship for a portion of the period of this investigation. A preliminary report was presented at the Ninety-fourth Annual Meeting of the National Academy of Sciences, April, 1957.

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† Samples of scopoletin used as standards had been kindly supplied by Drs. W. A. Andreae and R. J. Best to Dr. Katharine Tryon Bradley in this Laboratory.

‡ New data (Sargent and Skoog[7]) have shown that glucose alone from scopolin is the glucone and suggest that the spot corresponding to fructose here and in the cited work on *Avena* may have been due to chlorogenic acid present as a contaminant.

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NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY: ABNORMAL SPLITTING OF ETHYL GROUPS DUE TO MOLECULAR ASYMMETRY,†*

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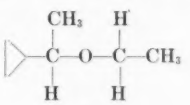
Nuclear magnetic resonance (n. m. r.) spectroscopy provides an excellent means for qualitative identification of ethyl groups by use of the familiar three-four pattern of spin-spin splitting.[1] It has been observed previously[2] that the methylene protons of systems of the type $R—CH_2—CR_1R_2R_3$ (where R_1 can be the same as R or different) may be magnetically nonequivalent and display AB rather than A_2-type spectra.[3] We now wish to report several examples of this type of behavior with ethyl groups, particularly ethoxy groups, knowledge of which could be important to anyone using n. m. r. for organic qualitative analysis.

A typical example is cyclopropylmethylcarbinyl ethyl ether (I). The spectrum of the ethyl CH_2 group of I is nearly identical to that of the CH_2 groups acetaldehyde diethyl acetal (II) shown in Figure 1 and appears not as a simple quartet



I

but as the very complicated AB part of an ABX_3-type spectrum with $\nu_A - \nu_B = 9.0$ cps and $J_{AB} = 9.4$ cps. The nonequivalence of the CH_2 hydrogens is due to the asymmetric center at the carbinyl group of the cyclopropylmethylcarbinyl moiety, which acts to favor one of the possible rotational conformations about the C—O bonds over the others.[2] Similar behavior has been noted with acetaldehyde

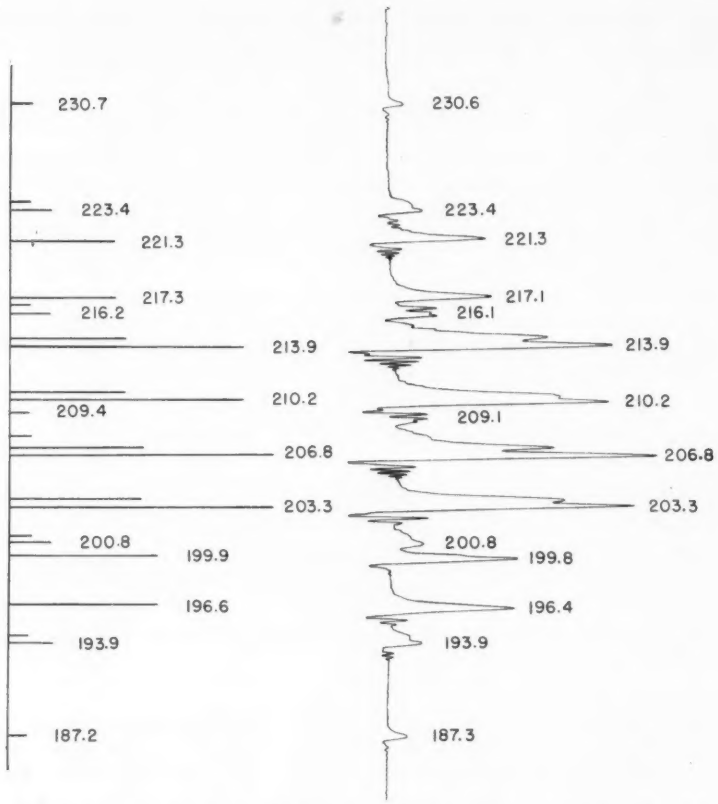
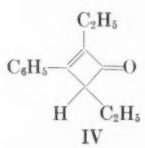


FIG. 1.—Observed and calculated nuclear magnetic resonance spectra of acetaldehyde diethyl acetal at 60 Mc with tetramethylsilane as internal reference. The calculated spectrum was based on an ABC_3 model with $\nu_A = 0.0$ cps, $\nu_B = 9.0$ cps, $\nu_C = 146.3$ cps, $J_{AB} = 9.4$ cps, $J_{AC} = 7.35$ cps, and $J_{BC} = 6.68$ cps.

diethyl acetal (II) and acetophenone diethyl ketal (III) and with ethyl-substituted cyclobutenones such as IV:

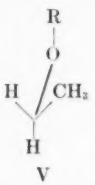


IV

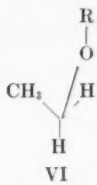
With IV, the effect is observed with the CH_2 of the ethyl located directly at the asymmetric 4 position and is further complicated of course by spin-spin interaction involving the 4 hydrogen.

The spectra of I, II, and III show a very interesting effect in that the methylene hydrogens of their ethyl groups are not equally coupled to the methyl hydrogens. Assignment of the respective couplings to be 7.35 cps and 6.68 cps for I and II

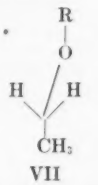
gives excellent agreement between calculated and observed line positions and intensities (see Fig. 1). Since the splittings result from electron coupling of nuclear spins, this speaks for induction of asymmetry in the bonding of the methylene protons to their carbon. Such would be expected to be the case if the effect of R were to make either V or VI a more favorable conformation about the C—O bond



V



VI



VII

than VII. Predominance of either V or VI would fit well of course with the considerable chemical shift difference between the methylene hydrogens. One of these conformations must be quite favored, since no significant change in the spectrum of II was noted at temperatures as low as —80°.

It seems possible that the asymmetry of the ethyl groups recently noted by Finegold[4] in the n. m. r. spectrum of diethyl sulfite is derived from the same causes as discussed above. We have noted that admixture of carbon tetrachloride with III results in changes of the methylene resonances in a manner analogous to that observed by Finegold for diethyl sulfite. Some other observations bearing on the relation of the present research to Finegold's results will be published later.

We are much indebted to Dr. Stanley L. Manatt and the Computing Center of the Jet Propulsion Laboratory for IBM 704 calculations of comparison spectra to check the chemical shift and spin-coupling parameters.

* Contribution No. 2640.

† Supported in part by the Office of Naval Research.

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*LINKAGE OF GENETIC UNITS OF BACILLUS SUBTILIS IN DNA TRANSFORMATION**

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Bacterial transformation, the transfer of genetic markers by DNA, provides a mechanism by which closely linked genetic loci may be ordered in a segmental map. Since a unit of DNA transferred from a donor bacterium to the recipient cell represents only a small fragment of the entire genome,[1] it is not possible to arrange many randomly distributed loci in any single transfer experiment. By the same token, when a pair of genes shows an appreciable degree of joint transformation, it points directly to their genetic contiguity. During a genetic and chemical study of the system of DNA transfer in *Bacillus subtilis* described by Spizizen,[2] it was observed that two markers could be simultaneously incorporated into a doubly auxotrophic recipient. DNA isolated from a wild type organism will transfer two independent characters—one concerned with an enzyme of tryptophan (more precisely indole glycerol phosphate) formation and the other with an enzyme of histidine biosynthesis. The evidence for their linked transfer is presented below.

Materials and Methods.—The origin and genotype of the principal strains employed in this study are given in Table 1. The liquid media employed include: Pen, Penassay Broth currently

TABLE 1

List of Strains of *Bacillus subtilis*

Strain	Genotype	Origin
168	*ind*$^-$	Burkholder and Giles[3]
23	*thr*$^-$	Burkholder and Giles
SB19	Reference prototroph	23 — × 168*
SB1	*ind*$^-$ his$_1^-$	UV treatment[4] of strain 168
SB25	*ind*$^-$ *his*$_2^-$	UV treatment[4] of strain 168
SB48	*his*$_3^-$	SB19 — × 168 (nitrous acid treatment)[5]
SB60	*ind*$^-$ *his*$_3^-$	168 — × SB48[5]
SB32	*his*$_2^-$	SB19 — × SB25
SB33	*ind*$^-$	SB19 — × SB25
SB31	Prototroph	SB19 — × SB25

* The symbol 23 —× 168 indicates that DNA from strain 23 was used to transform the recipient strain 168. SB31, SB32, and SB33 are transformants selected to insure an isogenic background for the *ind* and *his* markers.

sold as Difco Antibiotic Medium 3; CHT-2, minimal medium[2] containing 0.02 per cent acid hydrolyzed casein and 20 μg per ml of DL-tryptophan; CHT-10, minimal medium containing 0.1 per cent casein hydrolysate and 200 μg per ml of DL-tryptophan. For platings, we used minimal agar[6] (histidine agar or tryptophan agar) containing 40 μg per ml L-histidine or 20 μg per ml DL-tryptophan, respectively.

The recipient cells are grown aerobically[7] to the completion of exponential growth in Pen medium. The culture is washed and resuspended, 1:10 in a 15 × 125 mm test tube containing 5 ml of CHT-2 medium. After four hours of further incubation at 37°, the cells are washed and diluted 1:10 into fresh CHT-10 medium precooled to 30°, and 0.9 ml aliquots are dispensed into 15 × 125 mm test tubes. After incubation at 30° for 90 minutes, 0.1 ml of a solution of DNA (containing in most instances 1 μg per ml of DNA) is added to each tube, and incubation is continued for an additional 30 minutes. Deoxyribonuclease (Worthington Biochemical Corp.)

20 μg, and magnesium sulfate to give a concentration of 0.01 *M* are added. After an additional 10 minutes incubation, 0.1 ml aliquots of the cells are plated on the indicated minimal agar. These plates are incubated at 37°, and read at 40 to 48 hours. This procedure, a modification of the technique devised by Spizizen,[8] has given consistent results in our hands.

For the preparation of DNA, the donor cells are grown in Pen and washed twice in 0.14 *M* NaCl, and the packed cells are resuspended at a concentration of 1 gm (wet weight) per 4 ml of a solution containing 0.14 *M* sodium chloride, 0.01 *M* sodium citrate, and 0.01 *M* phosphate buffer solution, pH 6.6. Lysozyme (Armour) is added to give a concentration of 1 mg per ml and the suspension stirred at 37° for 10 minutes. The now viscous lysate is suspended in 10 volumes of a solution of 0.14 *M* sodium chloride and 0.01 *M* sodium citrate at 0°. The nucleic acid-protein complex is then sedimented at 0° at 30,000 × *g* for 20 minutes. The pellet is suspended in 4 volumes of 2 *M* NaCl relative to the original volume of the lysate and extracted by agitation with a magnetic stirrer for 30 minutes at 4°. The suspension is then centrifuged at 20,000 × *g* for 20 minutes at 0° and the pellet reextracted with 2 *M* NaCl. The pooled extracts are added to 2 volumes of 95 per cent ethanol previously cooled to −20°. The fibrous precipitate of DNA is collected on a hooked rod and resuspended in 2 *M* NaCl. After storage overnight at 4°, the DNA is deproteinized by gentle manual shaking with an equal volume of a chloroform-octanol (5:1) mixture for 10 minutes, followed by centrifugation at 2,500 × *g* for 5 minutes. The upper, aqueous layer is transferred to fresh chloroform-octanol and the process repeated (usually seven times) until no gelatinous interface between the two layers appears. The DNA is reprecipitated in 2 volumes of 95 per cent ethanol and dissolved in 2 *M* NaCl + 0.01 *M* phosphate buffer, pH 7.4. In this medium, the DNA has been stored for at least 6 months at 4° and has retained full transforming activity. However, repeated pipetting of the DNA solution often results in a loss in activity, perhaps due to shear forces.[9]

The procedure as described here is a modification of the procedures employed by Spizizen[2] and Ephrati-Elizur.[10] When prepared by this method, 0.02 to 0.05 μg of DNA will saturate 7×10^7 recipient cells in one ml.

Experimental Results.—Nonidentity of his mutants: The relationship of the single histidine markers to each other was studied by determining whether the DNA extracted from one *his*⁻ mutant could transform another *his*⁻ mutant to prototrophy (Table 2). If prototrophs do result, the mutant loci are recombinationally distinct, i.e., they do not mark the same point of the chromosome. The inference from Table 2 is that these three *his*⁻ strains carry nonallelic mutations.

TABLE 2

DNA TRANSFERS INVOLVING *his* LOCI

Donor DNA from	Recipient Bacteria SB1	SB25	SB48
SB1 (his_1^-)	0	60	86
SB25 (his_2^-)	21	0	15
SB48 (his_3^-)	33	31	0
SB19 (his^+)	226	98	190

The numbers are his^+ transformants per μl aliquot of recipient cell suspension (5×10^7 per ml). They were scored on tryptophan agar. The quantitative significance of the numbers is uncertain on account of possible variations in the competence of the recipient cultures and the absolute activity of the donor DNA.

Linkage: Although a comparison of the absolute frequencies of transformation to prototrophy could serve to estimate the relative distances of the mutant loci, as in Table 2, there are two important reservations: (1) the need to compare trials involving different, perhaps not comparable, batches of cells and of DNA and (2) the untested assumption that many transformants are produced with the donor markers which cannot be counted in the scoring system. A more reliable method depends on the actual recovery and estimation of single and multiple marker transfers.

The linkage relationship can be expressed as the cotransfer index, *r*, a measure

of the frequency of joint transfer of two markers compared to the total number of recombinant genotypes measured by the transformation experiment. To use a general notation,[11] in a system $a^1b^1 — \times\ a^\circ b^\circ$, giving transformant types a^1b^1, a^1b°, and $a^\circ b^1$,

$$r = \frac{a^1b^1}{a^1b^1 + a^1b^\circ + a^\circ b^1} = \frac{a^1b^1}{a^1 + b^1 - a^1b^1} \quad (\text{since } a^1 = a^1b^\circ + a^1b^1).$$

For example, in Table 3,

$$r = \frac{ind^+\ his^+}{ind^+ + his^+ - ind^+\ his^+}.$$

In other experiments, it may be possible to estimate a^1b^1 and a^1b° but not $a^\circ b^1$. In this case, we may assume $a^\circ b^1 \cong a^1b^\circ$ and write an estimated index

$$r_a = \frac{a^1b^1}{a^1b^1 + 2\,a^1b^\circ}.$$

For unlinked markers, r can be estimated as $a^1b^\circ/2N$, i.e., one-half the efficiency of transformation for either marker, N being the total number of (transformable) cells.

The ability of DNA extracted from wild type cells to accomplish the joint transformation of the three strains of *ind*$^-$*his*$^-$ cells to prototrophy is shown in Table 3.

TABLE 3

TRANSFORMATION OF *ind*$^-$ *his*$^-$ AUXOTROPHS TO PROTOTROPHY

Donor DNA		Recipient Cells	Transformant Classes, per 10^4 recipient cells: ind^+	his^+	ind^+ his^+ Found	ind^+ his^+ Calculated as random coincidence	Cotransfer Index r
SB31 $ind^+\ his^+$	— ×	SB25 $ind^-\ his_2^-$	70	72	48	0.5	0.51
SB31 $ind^+\ his^+$	— ×	SB60 $ind^-\ his_3^-$	3.9	5.0	0.03	0.02	0.003
SB31 $ind^+\ his^+$	— ×	SB1 $ind^-\ his_1^-$	50	100	0.8	0.5	0.005
SB32 $ind^+\ his_2^-$ / SB33 $ind^-\ his^+$	—×	SB25 $ind^-\ his_2^-$	46	20	0.2	0.09	0.003

The transformation procedure was as given in the protocols except that DNA was used at a concentration of 20 μg per ml for his_1 and his_2 and at a concentration of 0.1 μg per ml for his_3. The transformant classes were counted on minimal agar (for $ind^+\ his^+$), histidine agar (for ind^+) and tryptophan agar (for his^+), respectively. The calculated random coincidence is the product of $ind^+ \times his^+$.

The cotransfer index for the appearance of double transformants ind^+his^+ varies from 0.004 to 0.51 with different *his* mutants. This result is taken as evidence of linkage between the *ind* and his_2 loci. Introducing the ind^+ and his^+ markers on different DNA molecules by mixing DNA preparations from $ind^+his_2^-$ gave no evidence of linkage. This argues against the possibility that the linkage can be accounted for by the random coincidence of unlinked markers.

Additional auxotrophic markers have been introduced into strain 168 after UV irradiation or nitrous acid treatment of DNA *in vitro* or by selection of spontaneously occurring mutants as previously described. These markers included growth requirements for methionine, methionine plus lysine, glutamic acid or proline, valine plus isoleucine, cystine, and a streptomycin resistance marker. When limiting concentrations of SB19 DNA were used, the cotransfer index never exceeded 0.005. Thus, while *ind* is closely linked to his_2, it is not linked to any of six other markers.

Discussion.—The foregoing evidence indicates that the loci his_2 and *ind* stand in a special relationship to each other in contrast with several other pairs of loci studied in the same way.

The cotransfer index for these markers might be accounted for if the transformable cells were a very small fraction of the total. The estimation of competence by the addition of increasing amounts of DNA is not a satisfactory measure owing to the possible interference of one absorption with another. The main argument against this interpretation of the high cotransfer index for ind/his_2 is its unique value for these markers compared to other sets and to the introduction of ind^+ and his^+ from separate DNA preparations. The evidence is closely analogous to that for the linkage of the *Fla* and H_1 loci in Salmonella transduction[12] and for mannitol dehydrogenase and streptomycin resistance in Pneumococcus transformation.[1]

The present findings, in conjunction with those of Ephrati-Elizur *et al.*,[13] are the first evidence of linkage in *B. subtilis*. This type of analysis, hopefully, may open the way to more intensive studies such as have been done on the genetic chemistry of DNA in Pneumococcus on the one hand and on the sequential arrangement of genes and their physiological function in the enteric bacteria on the other. In fact, the histidine and tryptophan pathways have been studied especially extensively in Salmonella, where a striking correlation between linkage and biosynthetic sequence has been observed.[14] At first sight, such a correlation could not be inferred from the present data, but this question requires more complete biochemical and genetic study.

* This work was supported by training grant 2G295 and research grant C4496 from the National Institutes of Health and by a grant from the National Science Foundation.

† The work was done while the author was on a fellowship awarded by the American Cancer Society.

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*GENETIC ANALYSIS, BY MEANS OF TRANSFORMATION, OF HISTIDINE LINKAGE GROUPS IN BACILLUS SUBTILIS**

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Within the past few years, new approaches have been employed in microorganisms and bacteriophage systems for the study of physiological genetics and fine structure analysis of genetic loci.[1, 2] A detailed study of the correlation between genetic defects and enzymatic blocks in the histidine biosynthetic pathway was described recently in Salmonella,[3, 4] in which fine structure mapping of histidine-requiring mutants was constructed by means of transduction. This study, correlated with the study of enzymes involved in the biosynthesis of histidine,[4] suggested that the gene loci are linearly arranged in an order that corresponds to the sequence of the reactions they control.

The use of DNA preparations, by means of transformation, for genetic analysis offers an opportunity to study the relationship between the molecular structure of DNA and the genetic map. Such a study was recently described in Pneumococcus.[5]

The present investigation is concerned with the genetic study of linkage groups controlling the biosynthesis of histidine in *Bacillus subtilis* and their relationship to the indole locus, using as a tool the transformation phenomenon. Histidine-requiring (*his*$^-$) mutants were isolated when spores of *B. subtilis* were heated to a high temperature (155°) in the dry state in a vacuum.[6] Such mutants, as well as several others, were used in an attempt to analyze the genetic location of the sites involved. In addition, preliminary attempts were made to identify the corresponding biochemical blocks of such mutants.

Materials and Methods.—The parent strain of all the mutants used was the indole-requiring strain 168 *ind*$^-$ of *Bacillus subtilis*, kindly provided by Dr. J. Spizizen, Western Reserve University. From this strain, nine *his*$^-$ mutants were induced by heat (mutants 12, 13, 16, 17, 26, 30, 31, 32, and 35). In addition, four other *his*$^-$ mutants, induced by ultraviolet irradiation, were used in this study (No. SB1 and SB25, kindly provided by Dr. J. Lederberg, Stanford University, No. N-3, kindly provided by Dr. C. Anagnostopoulos, Western Reserve University, and No. R1, kindly provided by Dr. S. Riggsby, Yale University).

From strain 168 *ind*$^-$, a spontaneous back mutant to indole-independence was isolated (168 *ind*$^+$), and from this mutant, a sulfanilamide-resistant spontaneous mutant (168 *ind*$^+$/Sa) was isolated. Crude deoxyribonucleic acid (DNA), prepared from this strain, gave a high frequency of transformation from sulfanilamide sensitivity to sulfanilamide resistance.[7] This DNA was used to transform the previously mentioned *his*$^-$ mutants to sulfanilamide resistance (strains 12/Sa, 13/Sa, etc.).

Yeast extract (final concentration 0.002 per cent) was added to all minimal media.

Bacterial stocks were maintained on potato agar slants[8] stored at room temperature.

Bacterial extracts for enzymatic study: Bacteria were grown for 7 to 8 hours in minimal medium[8] supplemented with 0.5 per cent glucose, 5 μg of L-histidine, and 5 μg of L-tryptophan/ml. Harvesting the bacteria in the logarithmic phase was found to be critical for optimal enzymatic activity.[9] The enzymatic reactions were carried out as described recently.[10]

Biochemical methods: The cultures were grown in media indicated above and incubated with aeration for 72 hr at 37°. The cells were centrifuged and the supernatant fluids were tested for the presence of various metabolites. The Bratton-Marshall reaction[11] for detection of diazotizable

TABLE 1

TYPICAL RECIPROCAL TRANSFORMATION EXPERIMENT BETWEEN HISTIDINE-REQUIRING MUTANTS OF *B. subtilis**

Recipient strain	DNA Donor Strain R1/Sa			26/Sa			30/Sa			SB1/Sa			N-3/Sa			12/Sa			168 *ind*⁺/Sa		
	°Sa	H	H/Sa	Sa	H	H/Sa	Sa	H	H/Sa	Sa	H	H/Sa	Sa	H	H/Sa	Sa	H	H/Sa	Saw	Hw	Hw/Saw
R1	4,000	0	0	2,500	0	0	1,130	20	0.018	4,630	100	0.02	2,800	76	0.026	410	20	0.05	1,560	240	0.17
26	1,500	0	0	800	0	0	7,370	50	0.007	5,200	80	0.015	1,300	65	0.05				655	116	0.18
30	1,480	56	0.037	4,450	190	0.043	2,000	0	0	990	27	0.027	1,550	52	0.033	550	45	0.08	4,500	1,560	0.35
SB1	3,540	470	0.13	1,385	251	0.18	1,200	80	0.067	2,000	0	0	3,800	220	0.06	575	77	0.13	920	1,027	1.12
N-3	4,830	720	0.15	5,100	502	0.1	4,400	520	0.12	720	40	0.065	2,000	0	0	240	40	0.165	640	760	1.2
SB25	1,950	400	0.21	600	160	0.27	1,680	460	0.28	2,000	620	0.3	650	200	0.31	1,000	302	0.3	5,600	1,620	0.3

* The values listed represent total colony counts on duplicate plates or are calculated from a tenfold dilution.
°Sa Plated on minimal agar supplemented with 20 μg/ml of L-histidine, 20 μg/ml L-tryptophan, 250 μg/ml sulfanilamide.
H Plated on minimal agar supplemented with 20 μg/ml of L-tryptophan.

TABLE 2*

Recipient strain	13/Sa			SB25/Sa			31/Sa			32/Sa			16/Sa			168 *ind*⁺/Sa		
	Sa	H	H/Sa	Sa	H	H/Sa	Sa	H	H/Sa	Sa	H	H/Sa	Sa	H	H/Sa	Saw	Hw	Hw/Saw
13	1,300	0	0	3,400	0	0	1,024	10	0.01				8,400	1,380	0.17	3,750	1,400	0.35
SB25	5,000	0	0	2,000	0	0	10,800	78	0.008	840	111	0.13	2,100	408	0.19	5,600	1,620	0.3
31	7,800	100	0.012	6,080	50	0.008	2,000	0	0	835	170	0.1	730	310	0.18	1,250	334	0.27
N-3	740	1,000	1.3	530	620	1.2	1,100	1,350	1.2	260	157	0.6	880	820	0.95	640	760	1.2

* See Table 1 for details.

amines was applied in the presence and absence of acetic anhydride. The imidazoles were detected by the Pauly diazo reaction.[12]

Growth studies: Bacterial growth studies were performed by inoculating 10 ml portions of minimal medium (medium A)[13] supplemented with 0.5 per cent glucose and other appropriate nutrients in concentration of 5 μg/ml. The cultures were aerated at 37° for 18 hr and the growth pattern determined.

Preparation of crude DNA for transformation: Crude DNA was prepared as follows. Cells grown in 100 ml of Penassay broth (Difco) containing 0.5 per cent glucose (18 hrs, 37°, with shaking) were harvested and resuspended in 5 ml of buffer pH 7.4 (0.1 *M* NaCl, 0.014 *M* sodium citrate). 5 mg of crystalline lysozyme was then added and the suspension was incubated with shaking at 37° until cell lysis occurred and the suspension became viscous (15 to 20 min.) 0.2 to 0.25 ml of a 15 per cent solution of Duponol C was added, and the mixture was kept at room temperature for 10 min. The lysate was then added to 4 volumes of 95 per cent ethanol, the resulting fibers were washed in 70 per cent ethanol, resuspended in 2 ml of a 10 per cent NaCl solution, and stored in the cold for 18 hr. The solution was then homogenized and centrifuged; the supernatant fluid was then added to 2 volumes of 95 per cent ethanol; the resulting fibers were washed in 70 per cent ethanol and redissolved in 2 ml of 10 per cent NaCl solution. The total yield of such a preparation varied from 30 to 70 μg of DNA. The resulting crude DNA preparation retained its transforming activity for at least 2 months when stored at 4°.

The above DNA preparations from each *his*$^-$ sulfanilamide-resistant mutant, as well as from strain 168 *ind*$^+$/Sa were used in transformation procedures essentially as described previously,[8] using other mutant strains as receptors (reciprocal transformations).

Results.—Mapping of the various mutational sites was attempted by reciprocal transformation studies between the different mutants, assuming that the resulting number of histidine-independent (*his*$^+$) colonies is a function of the distance between the two recombinable sites: the one in the transforming DNA and the one in the DNA of a receptor cell. In order to avoid variables such as inevitable differences in concentrations or activities of various DNA preparations or differences in the competence of individual recipient cultures, an unrelated marker was introduced into the DNA-donor strains by transforming them with the DNA from strain 168 *ind*$^+$/Sa; such a marker served as a standard of reference.[5] Thus, the frequency of transformation to *his*$^+$ can be better evaluated if it is expressed as the ratio *H*/Sa, that is, the number (H) of *his*$^+$ colonies divided by the number (Sa) of sulfanilamide-resistant colonies (Tables 1 and 2). In the last columns of these tables are also listed the ratios obtained when DNA of the sulfanilamide-resistant prototrophic strain 168 *ind*$^+$/Sa was used for transformation. Such ratios (Hw/Saw) differ for different mutants used as recipients but are consistent for a given recipient. It is therefore necessary for comparison to express the trans-

TABLE 3

RECOMBINATION INDEX (RI) OF RECIPROCAL TRANSFORMATIONS BETWEEN HISTIDINE-REQUIRING MUTANTS*

Recipient strain	DNA Donor Strain											
	R1/Sa	26/Sa	30/Sa	SB1/Sa	N-3/Sa	12/Sa	13/Sa	SB25/Sa	31/Sa	32/Sa	16/Sa	17/Sa
R1	0	0	0.1	0.12	0.18	0.30	0.60	1	0.75	—	0.80	0.60
26	0	0	0.07	0.08	0.22	—	1	1	—	—	0.70	0.70
30	0.12	0.12	0	0.07	0.1	0.23	0.70	1	—	0.40	0.60	0.45
SB1	0.14	0.19	0.07	0	0.07	0.12	0.75	0.80	0.70	—	1	0.50
N-3	0.13	0.14	0.1	0.06	0	0.13	1	1	1	0.50	1	0.45
13	0.50	—	0.60	0.60	0.65	1	0	0	0.03	—	0.30	0.60
SB25	0.60	0.75	1	1	0.80	1	0	0	0.03	0.35	0.50	0.80
31	0.55	—	1	0.55	1	—	0.06	0.04	0	0.40	0.70	—

* Values are mean of 4–5 experiments with standard deviation not exceeding 30 per cent.

formation frequencies of individual mutants as the fractions of the frequency of transformation when DNA of the wild strain is used. Such value $\frac{H/Sa}{Hw/Saw}$ was recently termed the recombination index[5] (RI, Table 3).

Linkage among histidine mutants: As can be seen from Tables 1, 2 and 3, reciprocal transformations among the mutants R1, 26, 30, SB1, N3, and 12 yielded low numbers of *his*$^+$ transformants as compared with those obtained when the DNA's of strain 168 *ind*$^+$/Sa or strains 13/Sa, SB25/Sa, and 31/Sa were used. Low numbers of transformants were obtained again in reciprocal transformations among mutants 13, SB25, and 31. Strains 13 and SB25, as well as strains R1 and 26, seem to be either identical in their histidine mutational site or else too close to give *his*$^+$ recombinants. Strains 16, 17, and 32 do not appear to have their mutational sites close to those of any other mutants. Strains 16, 12, and 32 were not good recipients and could only be used as donors of DNA. Strain 35 merits special consideration and will be discussed below.

Linkage between histidine and indole mutants: During these studies, a number of *his*$^-$ *ind*$^-$ mutants yielded an unusually high frequency of double transformations to *his*$^+$ *ind*$^+$. To investigate this phenomenon further, experiments were designed to determine whether the high frequency of double transformants is a result of two independent recombination events within a single cell or whether it is due to a simultaneous transformation of the histidine-indole markers as a result of their closeness. In Table 4, the results are given of transformation experiments in

TABLE 4

SIMULTANEOUS TRANSFORMATION TO HISTIDINE INDEPENDENCE AND INDOLE INDEPENDENCE

Recipient strain	DNA donor strain	Number of Transformants			
		His	*Ind*	*His* and *Ind* (double)	Double/*His*.
13	13 (*his*$^-$, *ind*$^+$)* and 168 (*ind*$^-$)	126	157	0	0
	168 (*ind*$^+$)	400	450	260	0.65 ± 0.15 (5 experiments)
31	31 (*his*$^-$, *ind*$^+$)* and 168 (*ind*$^-$)	450	520	0	0
	168 (*ind*$^+$)	334	320	224	0.65 ± 0.20 (5 experiments)
30	30 (*his*$^-$, *ind*$^+$)* and 168 (ind$^-$)	800	1,000	0	0
	168 (ind$^+$)	1,000	1,140	30	0.03 ± 0.01 (3 experiments)

* DNA was extracted from a spontaneous back-mutant of the his$^-$ ind$^-$ strain to indole independence. The values listed represent total colony counts.

which two different DNA's, each capable of transforming one marker only, either histidine or indole, were incubated in the same tube. A second tube contained DNA from *his*$^+$ *ind*$^+$ strain. It can be seen that when histidine and indole markers are from two separate sources (two separate molecules), double transformations do not occur, whereas a high proportion of double transformants is obtained when the DNA comes from a single source; this finding suggests that the double transformation is simultaneous.

The results presented in Tables 3 and 4 make it possible to construct a partial map of the mutational sites of the histidine mutants studied (Fig. 1). On this map, the strains have been designated as follows: SB1, *his* 1; SB25, *his* 2; 13, *his* 4; 31, *his* 5; 32, *his* 6; R1, *his* 7; 26, *his* 8; 30, *his* 9; N-3, *his* 10; 12, *his* 12.

It can be seen that the data permit the arrangement of the mutational sites in a reasonable linear order, except for strain 31, whose position with regard to strains 13 and SB25 was arbitrarily mapped.

Strain 35: The following are the characteristics of this unusual strain. When tested for its requirements, it is *his*$^-$ *ind*$^-$. The results of transformation experiments with this strain are represented in Table 5. It can be seen that 100 per cent of double transformations for both markers were obtained not only when DNA from prototroph 168 *ind*$^+$ was used but also with DNA from another *his*$^-$ *ind*$^-$ strain. Conversely, the DNA from strain 35 behaves as the DNA from a prototroph in the transformation of other *his*$^-$ *ind*$^-$ strains by transforming both markers with a high frequency.

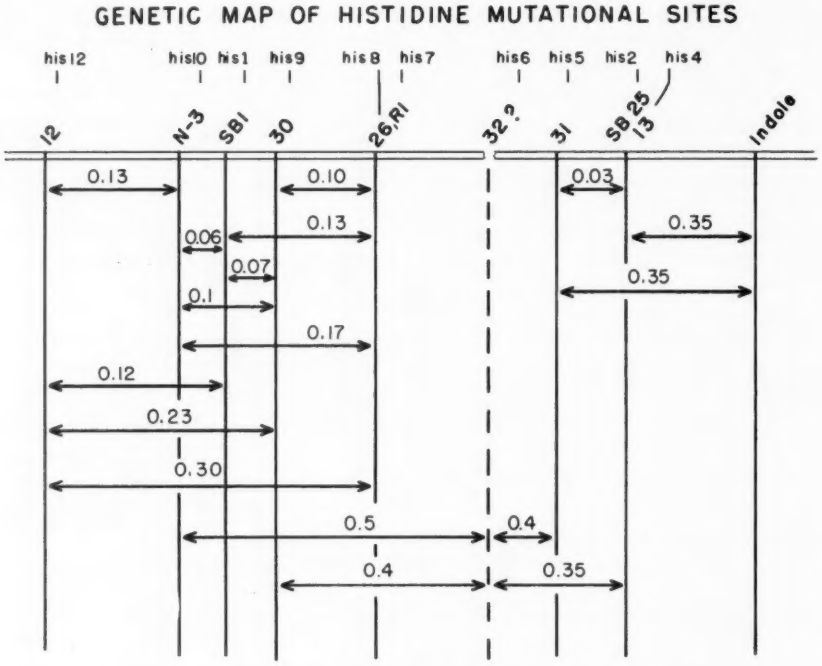

FIG. 1.—Genetic map of histidine mutational sites in *B. subtilis*. See text for explanation.

TABLE 5

TRANSFORMATION EXPERIMENTS WITH STRAIN 35

Recipient strain	DNA donor strain	Number of Transformants *His*	*Ind*	*His* and *Ind* (double)	Double as % of single transformations
35	168 ind$^+$	600	660	650	100 ± 20 (8 experiments)
"	SB25	221	200	244	100
"	31	145	135	136	100
SB25	35	950	...	550	57

The values listed represent total colony counts.

This strain reverts simultaneously to *his*$^+$ *ind*$^+$ at a very low rate; all the revertants tested were double revertants. Due to the unusual nature of this strain it could not be mapped with other *his*$^-$ mutants.

Biochemical studies: The biochemical block in strain 168 *ind*$^-$ was investigated by Spizizen[14] and found to be concerned with the reactions leading from anthranilic acid to the synthesis of indole glycerol phosphate. However, changes in the indole requirement were detected in some of the newly induced histidine requirers. As can be seen in Table 6, strain 12 can grow on histidine alone. Strain 32 also does not require indole but grows on histidine supplemented with shikimic acid.

TABLE 6

GROWTH REQUIREMENTS AND BIOCHEMICAL CHARACTERISTICS OF *B. subtilis* MUTANTS

Mutant strains	12	26	SB1	SB25	13	17	31	N-3	30	R1	16	32	35
Medium A	−	−	−	−	−	−	−	−	−	−	−	−	−
Medium A + histidine	+	−	−	−	−	−	−	−	−	+	−	−	−
Medium A + histidinol	±	−	−	−	−	−	−	−	−	+	−	−	−
Medium A ± histidine + anthranilic acid	+	−	−	−	−	−	−	−	−	+	−	+	−
Accumulation of anthranilic acid	−	+	+	+	+	+	+	+	+	−	+	−	+
Medium A + histidine + indole	+	+	+	+	+	+	+	+	+	+	+	+	+
Medium A + histidinol + tryptophan		+	+	−	−	+	+	+	+	+		±	+
Production of compound III* by cell-free extracts	+	+	+	−	−	+	−	+	+	+	+	+	+
Accumulation of Pauly-positive imidazoles	−	−	−	+	+	−	+	−	−	−	−	−	−

* See reference 10.

Preliminary experiments were conducted with the *his*$^-$ strains in an attempt to locate the biochemical blocks along the biosynthetic pathway of histidine. The results are presented in Table 6. It can be seen that strains 13 and SB25 do not grow on histidinol. Enzymatic reactions with cell-free extracts gave negative results when tested for Compound III or 5-amino-1-ribosyl-4-imidazole carboxamide 5′-phosphate[10] (ACP). Pauly-positive imidazoles were detected in the accumulation studies. Strain 31 behaves similarly to the above-mentioned mutants in the accumulation studies but it grows on histidinol. The remaining mutants grow on histidinol, form Compound III in cell-free extracts, and do not accumulate any Pauly-positive imidazoles.

Due to the preliminary nature of these studies, the location of the metabolic blocks in the histidine biosynthetic pathway is only tentatively suggested (Fig. 2).

Discussion.—Reciprocal transformations between *his*$^-$ mutants derived from various sources have shown that most of these mutants can be grouped into two distinct linkage groups. Although the biochemical studies are still at a preliminary stage, the few data obtained suggest that the two groups of linked mutational sites are correlated with at least two different biochemical reactions. From the present data, it is impossible to say whether these two groups of mutants happen to be situated on the same DNA molecule or whether the distance found between them is occupied by regions responsible for further steps in the histidine biosynthetic pathway (Fig. 2).

The data suggest that at least one group of mutants (12, 26, 30, SB1, N-3, R1) may be blocked at a region corresponding to the *A* region in Salmonella,[3] and that

the second group (13, SB25) may be blocked at the *D* region. The block in mutant 31 which grows on histidinol and does not form compound III may be tentatively located near the *D* region, an assumption based on the closeness of this strain to strain 13 and SB25 on the genetic map. However, extensive enzymatic studies with cell-free extracts will have to be carried out before the exact metabolic blocks of these mutants can be defined with assurance.

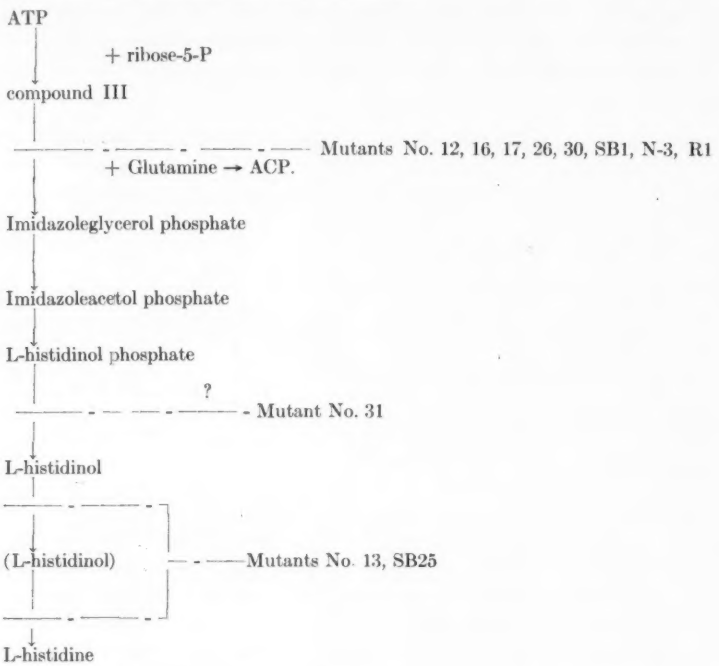

FIG. 2.—Tentative position of mutational blocks in the biosynthetic pathway of histidine (refs. 1 and 10).

The high proportion of double transformants *his*$^+$ *ind*$^+$ obtained in case of strains 13 (SB25) and 31 is of particular interest. This finding suggests that in these strains the mutational sites of histidine are close to the indole mutational site; these histidine sites might therefore be also close to each other, which is in agreement with the results obtained in the reciprocal transformation experiments.

Similar linkage studies are being reported by Nester and Lederberg[15] (this issue).

As a result of heat treatment of indole-requiring spores of *B. subtilis*, 9 *his*$^-$ mutants were isolated. In 3 of them, the heat-induced mutation was not confined to the histidine locus only. As mentioned above, in strain 12, the dependence on indole for growth disappeared, and this strain can grow on histidine alone. In strain 32, the mutation is no longer in the indole locus but appears to be connected with the synthesis of shikimic acid (Table 6).

In strain 35, the changes in the indole locus did not result in detectable changes in biochemical reactions, but there is genetic evidence that some change did occur

in the original mutational site in the indole marker. Although this strain requires indole for growth, it is able to transform with a high frequency other *ind*$^-$ strains to *ind*$^+$ (Table 5). This may suggest that the new mutational site is not close or linked to the original one. The simultaneous transformation of both histidine and indole markers, observed in all experiments, as well as the simultaneous spontaneous reversion of this strain to wild type, points to a possible functional relationship between the histidine and the tryptophane biosynthetic pathway.

It is hoped that more extensive studies with such mutants will lead to a better understanding of the correlation between DNA structure and function.

Summary.—Genetic analysis of histidine-indole-requiring mutants of *Bacillus subtilis* was attempted by means of transformation, using DNA preparations from these mutants. The position of the histidine mutational sites on the genetic map was based on the number of histidine-independent recombinants that were scored. Two distinct linkage groups were identified. One group was found to be closely linked to the indole marker.

Preliminary studies of the corresponding biochemical reactions in the histidine biosynthetic pathway suggest that one linkage group is concerned with the block at the region responsible for the formation of imidazole glycerol phosphate and the second one with the block at the region responsible for the reactions leading from histidinol to histidine.

The effect of heat on the mutation to histidine requirement resulted, in the case of a few mutants, in marked genetic changes in the indole region as well.

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THE DELAYED ORIGIN OF MUTANTS INDUCED BY EXPOSURE OF EXTRACELLULAR PHAGE T4 TO ETHYL METHANE SULFONATE

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Ethyl methane sulfonate is an alkylating agent which was discovered by Loveless[1] to induce mutations in bacteriophage T2 treated *in vitro*. The mechanism of this induced mutation has been investigated further, using the T4 r_{II} system. Exposure of T4 r^+ phage to 0.4 *M* ethyl methane sulfonate[2] for 60 min at 30°C inactivates approximately 50 per cent of the phage. The exposure is terminated by dilution, and when treated phage are plated directly with *Escherichia coli* strain B, about 1 per cent of the plaques appear to be *r* or "mottled" (plaques composed of a mixture of *r* and r^+ phage). This represents an increase of one hundredfold over the spontaneous frequency of these plaque types. When the presumptive *r* and mottled plaques are picked and replated, all are found to contain both r^+ and *r* phage. If the treated phage are adsorbed to strain B bacteria and allowed to undergo a one-step growth cycle in broth, the progeny phage are found to include an increased frequency of *r* type mutants but only a few "mottled" plaque formers.

The above results are generally similar to those reported by Loveless[1] and clearly show that the exposure of extracellular phage to the chemical induces mutations. However, from the experiments so far presented, we could not determine whether or not the mutagen reacted with the phage genes extracellularly. The mixed plaques observed in direct plating of treated phage indicated that the induced mutants appeared in bursts together with nonmutants. It remained nuclear whether the induced mutations occurred during the *in vitro* exposure or during the subsequent reproduction.

We considered three different general mechanisms, any of which could account for the appearance of induced mutants in mixed clones: (*a*) Induced mutation takes place in an infected bacterium "poisoned" by ethyl methane sulfonate which has been introduced into the bacterium with the treated phage but which has not yet reacted with phage genetic material. (*b*) Segregation occurs from phage in which only one of several copies of a gene has been mutated *in vitro*.[3, 4] (*c*) Delayed mutation occurs during the reproduction of treated phage whose genetic material has been altered extracellularly. Experiments to discriminate among and refine these hypotheses were performed and are presented in this paper.

Some of these experiments were made possible by the finding that ethyl methane sulfonate can induce "reverse mutations" in addition to "forward mutations."

The materials and methods used in this study are essentially those described by Krieg.[5] Synchronized infection of log phage bacteria was achieved through the use of cyanide in the adsorption mixture. The "single burst" technique and others are described by Adams.[6]

Test of Nonspecific Carry-Over of Mutagen.—The possibility of nonspecific carry-over of the mutagen (hypothesis A) was investigated by experiments involving mixed multiple infections of bacteria with treated and untreated phage. If and only if hypothesis A is correct and the treated phage carry in free mutagen, induced

mutants would be expected in the descendents of the untreated phage. The combination of an untreated revertible r_{II} mutant and a treated nonrevertible r_{II} mutant was chosen to allow efficient detection of reverse mutation to r_{II}^+ in the progeny of the untreated phage without confusion by mutations from the treated phage. The infecting phage types were incapable of giving rise to r_{II}^+ phage through recombination. The mutants employed were H88, a nonrevertible r_{II} mutant that defines a "segment" of the A cistron,[7] and Ap50, a revertible r_{II} point mutant that is included within this segment.[8] The nonrevertible r_{II} phage also carried a distinguishable plaque morphology marker which permitted estimation of the ratio of revertible to nonrevertible phage in mixed populations.

TABLE 1

INDUCED REVERTANTS AMONG PROGENY PHAGE FROM INFECTIONS OF TREATED AND UNTREATED PHAGE

	Infecting phage	Mean no. of viable adsorbed phage per bacterium	Mean burst size of progeny	Revertible/ nonrevertible phage in progeny*	Revertants ± std. error/ Revertible progeny phage†
1.	Untreated revertible	10.3	343	3.2	$3.9 \pm 1.9/10^7$
	Treated nonrevertible	3.4	370	—	$3.6 \pm 1.1/10^7$
2.	Untreated revertible	15.7	323	—	$245 \pm 30/10^7$
3.	Treated revertible	8.6			
4.	Treated revertible	4.4	277	0.36	$217 \pm 37/10^7$
	Untreated nonrevertible	12.7			

* The relative proportions of revertible and nonrevertible phage were measured by plaque types on *B/S* on which an *rI* plaque morphology marker carried by the nonrevertible phage could be distinguished from its wildtype allele carried by the revertible phage.

† The platings to determine revertants were made with 112-12 (λh), a bacterial strain which does not allow plaque formation by r_{II} phage. Platings for total phage were made with strains *B* and *B/S*. The efficiency of plating for r^+ phage on 112-12 (λh) was 0.91 of that on *B* or *B/S* in all infections listed here. No correction for efficiency of plating was made in the presented data. The standard errors for the revertant frequencies were determined from variance analysis of the plate counts.

Table 1 presents the results of one such experiment. The progeny of experimental and various control one-step growth cycles were examined. Table 1 shows that the incidence of revertants in the progeny of a mixed infection of treated nonrevertible and untreated revertible phage is not significantly different from the incidence of "spontaneous" revertants from untreated phage alone. Treatment of the revertible phage gives induced revertants to the extent of a seventyfold increase. Induced reversions occur in a mixed infection of treated revertible and untreated nonrevertible phage to an extent not significantly different from those found in an infection by treated revertible phage alone.

In this experiment, the mean number of adsorbed phage particles per bacterium was chosen to be about equal in each infection. The differing numbers of adsorbed viable phage reflect the inactivation (approximately 50 per cent) of the phage that were treated. The fate of inactivated phage in this system has not yet been investigated. In the mixed infections, the output ratio of revertible to nonrevertible phage did not change detectably from the input ratio in the adsorbed viable phage.

This experiment and others not tabulated indicate that mutation due to carryover of the mutagen in a nonspecific manner does not play any detectable role in the mechanism of ethyl methane sulfonate-induced mutation. Since the mutagenic action is manifest only in the descendents of the extracellularly treated phage, we may conclude that there is an *in vitro* interaction between the mutagen and the phage genetic material. The next step was to examine the events that take place

after the *in vitro* reaction in order to gain a clue as to the reasons for the appearance of mutants and nonmutants in mixed plaques.

Constitution of Individual Clones from Treated Phage.—Mixed clones due to normal segregation or delayed origin of mutants: Simple segregation of extracellularly mutated genes could account for a certain kind of mixed clones. It would require that the extracellular phage contain their genetic information in duplicate copies, only one of which is mutated. Both copies would be introduced into the same host bacterium, and if they segregate and both reproduce, approximately equal numbers of mutant and nonmutant descendants would be expected in the mixed clone. If, on the other hand, the extracellular reaction between a gene and the mutagen does not in itself mutate the gene, the origin of the mutant line may be delayed until after reproduction has begun. The longer the delay in the mutation, the more nonmutant copies of the gene would be produced first, and the smaller would be the fraction of mutant phage in the mixed clone. The examination of a number of mixed clones with respect to the fraction of mutants found in each was undertaken to see if they could be accounted for by normal segregation of extracellularly mutated genes or, if not, by some sort of delayed-mutation hypothesis.

Picked plaques: The first approach to the study of mixed clones was to plate treated r^+ phage and to pick plaques which recognizably contained r mutants. Thirty-four apparent r and "mottled" plaques were picked and sampled to determine the fraction of mutant phage in each. Twenty-eight had fewer than 50 per cent r phage, and in most plaques the mutants were a small minority. Thus, it appeared that normal segregation of preformed mutants could not account for the mixed clones found in ethyl methane sulfonate-induced mutation.

This technique had previously been used by Tessman[4] in a study of nitrous acid-induced mutation. He found that mottled plaques produced by that mutagen tended to have equal numbers of mutant and nonmutant phage; some plaques had slightly less than half mutants, but other plaques had slightly more.

The finding that ethyl methane sulfonate-induced mutants comprised small fractions of the mixed clones in which they appear was in qualitative agreement with the hypothesis of delayed origin of mutants, but this type of data was not further analyzed quantitatively, because plaques with a very small proportion of induced mutants might not be recognized as mottlers and because the proportion of r to r^+ phage in a mixed clone might be distorted in the growth of a mottled plaque.

Single-burst experiments: Quantitative analysis of the relative number of mutants in individual mixed clones was accomplished in experiments free of the limitations just mentioned in the study of picked mottled plaques. It involved the observation of mutant progeny phage in individual "bursts" produced by the lysis in liquid medium of bacteria infected with treated phage. Aliquots of bacteria infected with a low mean number of phage particles (so that most infected bacteria had adsorbed only one phage) were distributed to a series of "single-burst" tubes such that less than one-fifth of the tubes contained bacteria producing induced mutants. The tubes were incubated to allow phage reproduction and bacterial lysis, and the content of each tube was subsequently plated individually with appropriate bacteria. This procedure made possible detection of every burst which produced one or more induced mutant phage and enumeration of the mutants in

each mixed burst. One "single-burst" experiment involved induced mutations of r^+ to r, and another involved the induced reversion of Ap72, an r_{II} mutant, to r^+. A control distribution for spontaneous reversion was included in the second experiment. In the former experiment, both r and mottled plaques were scored as mutants. In the latter experiment, revertant progeny were scored by plating with 112–12 (λh) bacteria, on which r_{II} phage are not capable of forming plaques. In neither experiment did the experimental design permit the enumeration of non-mutant phage produced in isolated mixed bursts. Some details of these experiments are recorded in Table 2.

TABLE 2

CONDITIONS OF INFECTION AND DISTRIBUTION IN THE SINGLE-BURST EXPERIMENTS

Class of Mutant Scored / Input phage type		r and Mottled Plaques: Treated r^+		r^+(Plaques on 112—12(λh)): Treated r	Control r
Adsorbed viable phage per host bacterium*		0.28		0.01	0.01
Infected bacteria per single burst tube*	i	2	4	140	1825
Plates with mutants per total plates	m/t	13/165	57/338	91/892	48/483
Total mutant bursts, calculated	$b = t \log_e (t/t - m)$	13.2	62	97.2	51
Total mutant bursts per total bursts in single burst tubes	b/it	13.2/366	62/1352	$97.2/1.25 \times 10^5$	$51/8.8 \times 10^5$
Estimated number of plates with more than one mutant burst	$b - m$	0.2	5	6.2	3
Expected number of spontaneous mutants†		...	...	7.5	...
Total number of mutant plaques on single burst plates	p		1993	3412	163
Mean size of mutant bursts	$M = p/b$		26.6	35.1	3.2
Mean total burst size*	B		180	256	357
Mean mutant fraction in bursts with mutants	M/B		0.148	0.137	0.011

* Viable phage and bacteria preparations were assayed prior to mixing for adsorption, and unadsorbed phage or infected bacteria diluted through anti-T4 serum were assayed before dilution for the single burst distributions. Parallel with the dilution for the single burst distribution, another dilution containing a large number of infected bacteria was incubated and subsequently assayed to determine the mean total burst size. The infected bacteria of the r^+ to r experiment were distributed at the two concentrations indicated and the data from single bursts pooled.

† This value is determined directly for the r^+ reversion study from the control distribution of the mutations of spontaneous origin. However, for the induced r distribution this value is estimated indirectly from the occurrence of spontaneous r and mottled plaques in control platings of infected bacteria. Under these conditions, 4 mutant plaques were observed among 20,000 plaques examined. In view of the difficulty of scoring mottled plaques in which the mutant type is in the minority, this value should be increased. If spontaneous r mutants occur with a frequency tenfold greater than detected they would still constitute less than 5 per cent of the distribution.

If we assume that mutant bursts arise by clonal growth from single mutational events, it is possible to classify each mutant clone according to the number of generations of binary reproduction that would be required to produce a clone of that size. The data, pooled into classes beginning with powers of two, are presented in Table 3. The "raw data" and the tabulated distributions permit the following conclusions: (1) In each experiment, mutant clones range in size from 1 to nearly the mean total burst size measured in an accompanying mass lysate. (2) The mean number of induced mutants in a burst is about one-seventh of the mean total burst size. (3) In general, there are about equal numbers of induced mutants in each size class—a distribution distinctly different from that found for spontaneous

TABLE 3

NUMBER OF MUTANT CLONES OF DIFFERENT SIZE CLASSES IN SINGLE-BURST EXPERIMENTS

Size class*	Clone sizes included	Ethyl methane sulfonate induced r^+ to r	Ethyl methane sulfonate induced r to r^+	Spontaneous r to r^+
0	1	15	24	38
1	2–3	10	11	6
2	4–7	7	5	2
3	8–15	10	12	1
4	16–31	12	10	0
5	32–63	7	12	0
6	64–127	7	11	1
7	128–255	1	5†	0
8	256 or greater	1	1	0

*Size classes are numbered according to $\log_2$ of the smallest member of the size class; this is a function of the number of generations from the end of growth in which the clone orignated.

†One of the clones of this size class was seen to consist of two plaque types in approximately equal numbers, 138 r^+ and 103 smaller than usual plaques. Analysis of the smaller plaque showed it to be of the genetic constitution r^+ and *minute;* the minute modifier being unlinked to the r^+ reversion.

mutation.[9] In the distribution of induced r mutants, the probability that the numbers of mutant clones in the classes 0–6 are random fluctuations from their mean is between 0.5 and 0.3. In the distribution of induced reverants, the numbers in classes 1–7 do not significantly differ at the 0.3 level. In this distribution, however, an excess of clones with only one mutant, i.e., of "class zero" is found, suggesting that this class is about twice as large as the others. Chi-square tests were made to evaluate the relative likelihoods that the number of clones in the class zero is one-eighth or two-ninths of the total number of clones in classes 0–7. Whether or not the expected number of spontaneous clones is subtracted from the observed data, the distribution is in excellent agreement with the interpretation that the "class zero" is twice as large as the other classes. The number of clones found in the "class zero" when corrected for mutants of spontaneous origin would be expected with a probability of only 0.02–0.01 if it were interpreted as a chance fluctuation from equality with the other classes. The fact that we did not detect a significant excess of class zero clones in the induced r distribution may be attributed to the lower efficiency of distinguishing mottled plaques from r^+ plaques, but even here, a chi-square test shows that the data do not contradict the assumption that class zero is twice as large as the other classes.

If we grant the assumption that established mutant phage undergo binary reproduction at the same rate as do nonmutant phage, the distributions of induced mutant clone sizes appear consistent with an hypothesis that the act initiating the mutant clone has an equal probability of occurring in any phage generation. This mode of production is in contradistinction to that found for spontaneous mutation,[9] since in spontaneous mutation the act giving rise to the mutant clone occurs randomly with a constant probability per phage particle per generation. Thus, in the earlier generations where the numbers of duplicating phage are small, the probability for a spontaneous mutation is much lower than in later generations.

One explanation of the excess of class zero clones could be that there are actually two generations in the growth of a mutant clone that are represented by single mutant individuals. This would be the case if the mutation produced a heterozygote which then underwent a reductional division to produce a homozygous mutant and a homozygous nonmutant. Heterozygotes are known to be capable of producing plaques under the conditions used to assay revertants.[10, 11]

Other studies of induced mutation have indicated that the genes of mature T4 phage are each present in two copies[3, 4, 11] and that a mutagen reacts with individual copies of genes during extracellular treatment.[3, 4] Our data also support the inference that the induced mutant clone descendant from an extracellularly treated phage in its first bacterial infection comprises not more than half of its total progeny phage. Direct measurement of the maximum fraction of a mixed burst that may be mutant is complicated in our experiments by the fact that we do not observe nonmutant members of the mixed burst in isolation from other pure nonmutant bursts and by the fact that the total size of individual bursts is normally quite variable. However, calculations have been made from the total data which do provide an answer to the question.

We assume, as the previous analysis has indicated, that there are an equal number of mutant clones in the various size classes from class 1 to the maximum. The ratio of the mean mutant clone size to the mean total burst size (M/B) therefore can be predicted on the basis of alternative hypotheses concerning the maximum mutant clone size. The approach is illustrated for a simple model in which synchronous complete maturation is assumed to occur after n generations of binary duplication, giving a total burst size of 2^n, and in which mutant clones comprised of a single phage (class 0) occur twice as frequently as clones of the other size classes. If the maximum mutant clone size is equal to the total burst ($M_m = B$), then it may be shown that

$$M/B = \frac{1 + 1/2 + 1/4 + \ldots + 1/2^n + 1/2^n}{1 + n + 1} = \frac{2}{n + 2}.$$

Similarly, if the maximum mutant clone size is half the total burst ($M_m = B/2$), then $M/B = \dfrac{1}{n + 1}$, and if the maximum mutant clone size is one-fourth the total burst ($M_m = B/)4$, then $M/B = 1/2n$. A model has also been analyzed which more nearly corresponds to known facts of phage reproduction; it assumes that beginning with the $k + 1$ generation of binary duplication, each vegetative phage may mature with a probability of one-half prior to its next duplication.[12] Thus, there are m generations of maturation in which a constant mean pool size, 2^k, of vegetative phage is maintained. Predictions based on this model have been calculated for various subsidiary assumptions as to M_m, m, k, and the relative size of class 0. The various predictions are compiled in Table 4. It is clear that the values of M/B observed in the single burst experiments (0.148 and 0.137 for the forward and reverse mutations, respectively) accord well with the assumption that the maximum class of mutant clones regularly produced is half the total burst size. It is also evident that M/B is relatively insensitive to the alternatives considered for the relative size of class 0, and k and m.

It may be pointed out that the action of this mutagen is similar for all the recognizable mutable sites, since r^+ to r distribution (which represents mutations at any of a number of mutable sites within the r_{II} cistrons and other r loci of the phage genome) is not significantly different from the r to r^+ distribution (which is presumably generated by reversions at a single mutable site).

The finding that a pure mutant clone may be initiated with an equal probability in any generation implies that the original altered gene retains its unique muta-

bility, even though it may make normal copies not highly mutable and persists at least until the initiation of a mutant clone.

Interpretation.—The interpretation in molecular terms of delayed origin of mutant clones can be approached in terms of two events: (1) the original alteration of the genetic material by the extracellular exposure to the mutagen, and (2) the secondary event by which the altered gene initiates a mutant clone. Three formal alternatives will be described as to the nature of the secondary event.

A. A pool of reproducing phage is set up containing genes derived from the unaltered allele of the altered gene, and the secondary event is the entry of the altered gene into the reproducing pool. This may be referred to as a "delayed reproduction" hypothesis rather than "delayed mutation," since the secondary event need not further alter the structure of the genetic material at the site of the chemical alteration. The delayed entry could be attributed to genetic recombination and the phenomenon related to "marker rescue" or "cross-reactivation." It might seem surprising that the secondary event would occur with standard probability even at the first duplication, before the pool of reproducing phage reaches full size. For this and other reasons, we do not regard this hypothesis as attractive, but further tests of its plausability are desirable.

The other two hypotheses each fit the definition of delayed mutation and assume that the original alteration does not impose a special block to the reproduction of the altered gene relative to its unaltered allele.

B. The original alteration of the genetic material may leave it in an unstable condition, still capable of making normal copies, but subject to an irreversible change generating a mutant clone. For instance, if the ethylation of a purine nucleotide did not alter its base-pairing properties but made it unusually susceptible to hydrolysis of the purine from the deoxyribose moiety, the secondary event may lead to a mutant clone in which the corresponding base had not been specified by the normal pairing rules. It is not obvious, however, that mutations produced by such a mechanism would be capable of spontaneous reversion nor that reversions of some mutants could be produced by this mechanism. A systematic study of possible specificity of induced reversion of various r_{II} mutants is under way which may bear on this question.

C. The secondary event may be a "copy-error" on the altered gene due to a reversible change of its molecular configuration. For instance, if the ethylation of a nucleotide increased the probability of its being in an otherwise very rare tautomeric state at the time of its action as a template for synthesis of new genetic material, a clone of phage with a particular kind of base pair substitution could be initiated. The reversibility of this tautomeric state would imply that at the next duplication the altered base would again pair with its normal rather than the "copy-error base"; this would predict that the mutant clone would be initiated by a heterozygote, as suggested earlier. A model rather similar to this has been presented for some spontaneous mutations.[13]

Summary.—Mutants induced by exposure of extracellular phage T4 to ethyl methane sulfonate are found in mixed clones; i.e., the treated phage individuals that have mutant descendants also have nonmutant descendants. Several underlying mechanisms of induced mutation that could generate mixed clones are considered and evaluated.

TABLE 4

PREDICTED VALUES OF MEAN MUTANT FRACTION, M/B

Model I: Maturation synchronous and complete after n generations.

n	Burst size	Class O equal to other classes		Class O double the other classes	
		M_m* = B	$M_m = B/2$	$M_m = B/2$	$M_m = B/4$
7	128	0.250	0.142	0.125	0.0715
8	256	0.222	0.124	0.111	0.0625

Model II: Maturation beginning after k generations and continuing for m generations.

k	m	Burst size	Class O equal to other classes		Class O double the other classes	
			$M_m = B$	$M_m = B/2$	$M_m = B/2$	$M_m = B/4$
5	5	160	0.251	0.143	0.126	0.072
5	8	256	0.250	0.143	0.125	0.071
6	2	128	0.234	0.133	0.121	0.069
6	3	192	0.228	0.128	0.116	0.065
6	4	256	0.225	0.126	0.113	0.064

* M_m symbolizes the maximum class of mutant clone sizes.

When bacteria are infected by treated phage, no free or reversibly bound mutagen can be detected that is capable of mutating genes which had not previously been exposed to the mutagen.

The relative proportions of mutants and nonmutants in the mixed clones do not correspond to a simple segregation from phage that contained an extracellularly mutated gene and a nonmutant allele. The number of mutants occurring in the mixed clones and the frequency distribution of these numbers indicate that a mutant clone may be initiated with an equal probability in any generation of vegetative phage growth.

Two types of alternatives remain. In one, it is assumed that the mutation is stabilized prior to reproduction so as to generate a pure mutant subclone and that the nonmutant members of the mixed clone are all descendant from an unaffected allele also present in the treated phage; it also requires the assumption that the reproduction of the mutant gene is delayed relative to its nonmutant allele. The other is based on a concept of "delayed mutation": it assumes that an initial reaction between the gene and the mutagen, occurring during the extracellular exposure, is prerequisite for mutation, but that the mutation itself occurs during subsequent reproduction. The distinguishing feature of this hypothesis is that the affected mutable site is assumed to be capable of reproduction prior to the establishment of a mutant clone.

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*COMPLEMENTATION BETWEEN ALLELES OF THE Td LOCUS IN NEUROSPORA CRASSA**

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During the past ten years, a large number of mutants of *Neurospora crassa* have been isolated which are unable to catalyze the final step in tryptophan synthesis.[1, 2] Despite the similarity of these mutants as judged in terms of their nutritional requirements, loss of enzymatic activity, and location of altered genetic sites,[1] they differ from each other when judged in terms of temperature sensitivity,[1] accumulation of indole,[2] response to specific suppressor genes,[1] and ability to form CRM (cross-reacting material), a protein immunologically related to the enzyme.[3] These differences clearly indicate that the locus involved is mutationally highly complex. In fact, it would appear that none of the more than 100 mutants now isolated is truly identical with any of the others.

In view of the detailed information available concerning the functional differences among these allelic strains, it was desirable to determine whether or not these strains could exhibit complementation when combined in heterocaryons. If such proved to be the case, then it should be possible to construct a complementation map[4] of the locus and to observe any correlations existing between the grouping of the mutants by complementation responses and their already known functional differences.

The present paper will deal with the complementation pattern of this locus—which controls the formation of the enzyme tryptophan synthetase in *Neurospora crassa*. This enzyme catalyzes the terminal step in tryptophan synthesis: an exchange reaction of indole-3-glycerol phosphate (IGP) with serine to form tryptophan.[5, 14] It has also been shown that this enzyme can catalyze a reaction involving the conversion of IGP to indole and a reaction of indole with serine to form tryptophan.[5, 14]

Materials and Methods.—All of the *Td* mutants used in these experiments, with the exception of *td* 1 and *td* 2, were isolated from the St. Lawrence wild type strain 74*A*, or its derivatives, by the filtration technique of Woodward *et al.*,[4] following ultraviolet or X irradiation of macroconidia.[2] Many of the mutants were extensively back-crossed to wild type 74*A*, and isolates were selected which exhibited both heterocaryon compatibility and fertility in interallelic crosses.[2]

Tests for complementation in heterocaryons were carried out by a method similar to that of de Serres.[7] Drops of conidial suspensions of each of two mutant strains were pipetted onto a minimal agar plate in such a way as to have control spots of

the two strains as well as a mixed spot containing both strains. Combinations were tested in duplicate and incubated at 30°C (25°C if a temperature-sensitive mutant was involved). Any combination which did not exhibit more growth after 14 days incubation than did the control spots was considered to be negative in terms of complementing ability. Minimal Medium *N* (Vogel)[8] was used throughout this investigation.

In crosses between *Td* mutants, pseudo-wild type progeny[9] were distinguished from true wild type progeny by a difference in colony morphology and a later time of colony appearance. The validity of this method had been previously established by crossing such progeny by a known wild type strain and recovering both mutant and wild type progeny.

The crude extracts of *Neurospora* tested for tryptophan synthetase activity were prepared as described by Suskind[14] and the enzyme was assayed by the method of Yanofsky.[11]

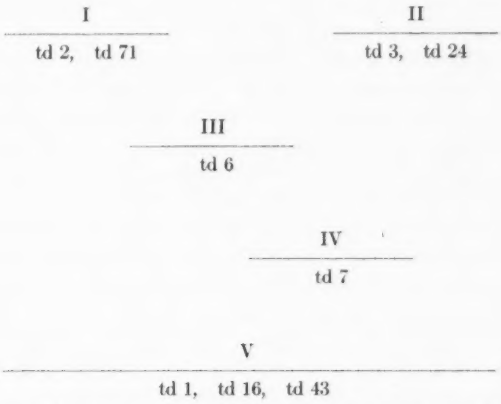

FIG. 1.—Complementation Map of the *Td* Locus. *Td* mutants can be divided into five groups on the basis of complementation responses in heterocaryons. The relationship between these groups is expressed above in terms of a complementation map in which overlapping bars indicate a lack of complementarity. Mutants typical of each group are indicated below each bar.

Results.—In this study, 75 *Td* mutants were tested for complementing ability. While 62 were tested in all combinations, 13 others were tested only with selected strains. Of the 75 mutants, 18 were able to complement at least one other *Td* mutant. Of these, 12 mutants can be divided into four different complementing groups on the basis of their responses in heterocaryons. The other 6 complementing mutants cannot yet be classified since their ability to complement is sporadic. A large fifth group is composed of the 57 apparently noncomplementing mutants. In Fig. 1, these five groups are arranged in a diagrammatic way to form a complementation map similar to that designed for the *ad-4* locus by Woodward *et al.*[4]

In crosses between many of these *Td* mutants (the detailed results of which will be presented elsewhere[12]), some pseudo-wild type progeny as well as true wild type progeny were obtained. As might be expected, there was a close correlation between the occurrence of pseudo-wild type progeny and the complementing ability of the two parents when combined in a heterocaryon.

A cross of *td* 7 × *td* 71 was the only cross between mutually complementing mutants which did *not* appear to yield any pseudo-wild type progeny. In this case, the nonappearance of such progeny may be attributable to the very slow growth of the *td* 7-*td* 71 heterocaryons formed from pseudo-wild progeny, which prevented the detection of the colonies before termination of the experiment.

Characteristic of all the complementing *Td* mutant strains is the fact that functional heterocaryons between them form slowly and that the growth of these heterocaryons on minimal medium is far slower than that of the wild type strain. Forced heterocaryons were formed (on tryptophan supplemented medium) between complementing *Td* mutants by the use of additional biochemically unrelated markers. This technique did not result in an increased growth rate of the heterocaryons following transfer to minimal medium.

At the present time, only two interallelic *Td* heterocaryons have been examined for the presence of tryptophan synthetase activity measured in terms of the ability to catalyze the reaction of indole and serine to form tryptophan. A *td* 6–*td* 3 heterocaryon was found to exhibit about 5 per cent of the tryptophan synthetase activity characteristic of the wild type strain grown under the same conditions, while a *td* 71–*td* 24 heterocaryon (grown at 25°C) yielded about 18 per cent of that of the wild type. It was found that the enzyme formed by the *td* 71-*td* 24 heterocaryon is more sensitive to zinc than is that formed by the wild type strain.[2]

Discussion.—Previous studies of the *Td* locus in *Neurospora crassa* have clearly indicated that this genetic region which controls the formation of tryptophan synthetase is highly complex in terms of mutation, recombination, and function.[1, 2] This complexity has permitted inquiry into the question of whether or not the phenotypic differences found among allelic *Td* mutants are reflected in the complementation pattern of the locus.

It is immediately apparent that those mutants which are able to complement exhibit other interesting characteristics (Table 1). One trait shared by all of the

TABLE 1

FUNCTIONAL CHARACTERISTICS OF TD MUTANTS

Characteristics	Groups I	II	III	IV	V
Temperature sensitivity	−	+	−	−	−
Indole accumulation	+	−	−	−	−
Suppressibility	+	+	+	−	−
CRM formation	+	+	+	+	−
Representative Td mutants	td 2 td 71	td 3 td 24	td 6 td 43	td 7	td 1 td 16

Td mutants can be divided into five major groups on the basis of the four criteria listed above. Mutants representative of each group are listed on the bottom lines. A number of the *Td* mutants have not yet been classified as to whether they belong in groups III, IV, or V. *Td* 43, for example, has not been tested for response to known suppressor genes.

complementing mutants tested is the ability to form CRM, a protein immunologically related to the tryptophan synthetase formed by the wild type organism.[13] Although not all CRM-forming *Td* mutants are able to complement, no case has yet been observed in which a non-CRM-forming *Td* mutant is capable of complementation. Perhaps a prerequisite for complementation at this locus is the ability of each of the mutants involved to form a protein which is closely related to the wild type enzyme.

A consideration of other characteristics of the complementing mutants seems to indicate that, in general, those mutants which are closely related functionally cannot complement each other, while those mutants having different types of functional alterations are often capable of complementation. For example, mutant strains *td* 2 and *td* 71 both accumulate indole[2] and have been shown to form CRM's which are enzymatically active in catalyzing the formation of indole from IGP.[14] These two strains cannot complement each other, but either strain will complement *td* 3 or *td* 24, neither of which accumulates indole.[2] Strain *td* 24 is temperature-sensitive and suppressible.[1] *Td* 3 is suppressed by the same suppressor as *td* 24, and in this condition, it is also temperature-sensitive.[1] Both *td* 24 and *td* 3 *su* 3 form tryptophan synthetase which is more zinc-sensitive than is that of the wild type organism.[14] Thus, the mutational alterations of *td* 24 and *td* 3 would appear to be similar and the two strains, as one might expect, do not complement each other but do complement *td* 2 and *td* 71.

Strain *td* 6 is not temperature-sensitive[1] and does not accumulate indole[2] but would appear to be related to strain *td* 2, since it can be suppressed by one of the same suppressors.[1] *Td* 7 does not accumulate indole,[2] is not temperature-sensitive,[1] and is not suppressed by any known suppressor;[1] thus, its functional relationship to the other mutants is unknown. Of these 6 mutants, then, *td* 2 and *td* 71 will complement *td* 7, *td* 3, and *td* 24, but not *td* 6. *Td* 3 and *td* 24 will complement *td* 2, *td* 71, and *td* 6, but not *td* 7. The other 6 complementing mutants which can be classified would appear to belong in the same group as *td* 6 on the basis of their complementation responses. It has been shown that these mutants, also, do not accumulate indole[2] and are not temperature-sensitive.[2] (Their other characteristics are currently under investigation.) All these observations suggest that mutants exhibiting similar functional characteristics tend to have similar complementation responses.

Conventional heterocaryon tests indicate the existence of 4 complementing groups. However, the recovery, in preliminary studies, of what appear to be rare pseudo-wild type progeny from crosses of *td* 6 × *td* 7 suggests that the *Td* locus may comprise only 2 *major* functional regions; i.e. it appears possible that only two major complementing groups may be found.

The possible division of these complementing *Td* mutants into 2 major groups is of particular interest in view of the observation of Crawford and Yanofsky[16] that the tryptophan synthetase of *E. coli* is dissociable into two protein components. The suggestion might be made that complementation phenomena in the case of the *Td* locus of Neurospora reflect a bipartite nature of the enzyme. However, present evidence does not support the view that the tryptophan synthetase of *Neurospora crassa* is dissociable.[14] It would appear unlikely, then, that complementation in this instance can be accounted for simply as enzymatic dissociation and recombination of the type observed by Singer *et al.*,[14] to occur in the hemoglobin molecule.

Co-polymerization has also been suggested as a possible mechanism of complementation.[19] However, this explanation does not appear to be favored by the evidence accumulated in the study of the *Td* locus.

Perhaps complementation between these *Td* mutant strains does not always involve interaction at the level of protein synthesis. A reactivation process might

be involved in some cases. We have already mentioned the temperature-sensitive mutant, *td* 24. This mutant when grown at 25°C (a temperature at which it exhibits its mutant phenotype) forms a CRM which can function as active enzyme after fractionation or dialysis.[15] This purified active enzyme can be shown to be more zinc sensitive than tryptophan synthetase formed by the wild type organism grown under the same conditions.[15] When *td* 24 is grown at 35°C or when *su* 24 is introduced into its genome, this strain can grow on a minimal medium as a result of the formation of active enzyme. When extracted and tested, this enzyme is found to be as sensitive to zinc as that formed when *td* 24 is incubated under conditions which will not permit its growth on minimal medium. Thus, *td* 24 appears to form a zinc-sensitive tryptophan synthetase, which under certain cellular conditions may remain enzymatically active, while under other conditions this enzyme is altered to CRM.[14] It could be postulated that, in complementing heterocaryons involving strain *td* 24, the functional enzyme is formed by the *td* 24 component, while the CRM formed by the other component strain would act in some way to free the *td* 24 enzyme from intracellular inhibition, perhaps through a greater affinity for zinc. A preliminary test of the enzyme formed by a *td* 71–*td* 24 heterocaryon seems to indicate that this enzyme is, indeed, more sensitive to zinc than that formed by the wild type organism grown under the same conditions.[2]

At present, the mechanism of complementation is not known and the explanation awaits a better understanding of the nature of the active enzymes formed through complementation in heterocaryons. The recent work of Fincham on a hybrid enzyme formed by a heterocaryon composed of two different allelic *am* mutants is a start in this direction.[24]

An additional point of interest is the possible relationship between the complementation map presented here and a recombination map of the same locus.[12] Since in both of these maps mutant strains can be arranged in a linear sequence, it is of interest to determine how close a correlation exists between the placement of alleles on each of them. This problem has been examined in considerable detail at the *pan-2* locus of *Neurospora crassa* by Case and Giles.[21] They conclude that there is good over-all correspondence between the two maps although there are instances on their maps in which the placement of alleles is not identical. A similar situation exists for the *Td* locus, although here instances have been noted in which complementation can occur between two alleles and yet no genetic recombination between these alleles has been detected.[12] This may indicate that the complementation map is not necessarily a reflection of the genetic map.

Summary.—The complementation characteristics of 75 *Td* mutants of *Neurospora crassa* have been examined. These mutants were found to comprise five complementation groups.** These mutants may also be divided into five generally corresponding groups on the basis of previously determined functional characteristics, suggesting a relationship between functional alterations and ability to complement in a heterocaryon. It was also noted that the ability to form a CRM, a protein immunologically related to the enzyme, appears to be a prerequisite for complementation in the *Td* system.

The significance of these observations was discussed in terms of possible mechanisms of complementation and of the relationship between structure and function of the region controlling the formation of tryptophan synthetase in *N. crassa*.

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** Catcheside and associates,[22] studying *Td* mutants isolated from a different wild type strain of Neurospora, have recently determined six complementation groups. One of these groups is represented by a mutant which can form tryptophan from serine and indole, a type of mutant not considered in this paper. The precise relationship of their mutants to those used in the present study is being investigated.

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*GENETIC CONTROL OF DOPA OXIDASE ACTIVITY IN DROSOPHILA MELANOGASTER, II. REGULATING MECHANISMS AND INTER-AND INTRA-STRAIN HETEROGENEITY**

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Much and perhaps most of the genetic information in the genomes of higher organisms is concerned with quantitative traits. Inherited quantitative differences, determined by many genes, are responsible in large measure for the distinction of individuals in a population and from an evolutionary point of view are probably more significant than qualitative differences determined by single genes. Due to the complexity of the genetic basis of quantitative traits, there is little experimental evidence concerning their genic background and control. A biochemical phenotype, such as enzymatic activity, is less complex than a phenotype at the morphological level, presumably is controlled by fewer genes, and has fewer steps between the primary genetic events and the observed phenotypes. It has previously been suggested[1] that dopa oxidase activity in *Drosophila melanogaster* may be considered a quantitative trait and that analysis of this system may provide information about the control and regulation of inherited quantitative differences at a molecular level. The evidence now presented shows that dopa oxidase activity in Drosophila is indeed a quantitative trait and that there may be two mechanisms operative: major controlling genes and regulating factors. These findings are discussed in relation to phenomena in microorganisms where the activity of a single enzyme is controlled by a number of interdependent genetic variables.[2–4]

Materials and Methods.—The dopa oxidase activity recorded in Tables 1 and 2 was measured in extracts of progeny from multiple matings; all other measurements of enzymatic activity were made on extracts prepared from the progeny of single-pair matings. Activity in homogenates of twenty flies or more can be reliably assayed. Flies up to 24 hrs. old were collected, frozen and stored under liquid nitrogen up to five days before the extracts were prepared according to the procedure described elsewhere.[1] In the survey of common laboratory strains, length of lag period and time of half-maximum activity were determined from activation curves obtained by assaying samples of extract during the activation process at 20-minute intervals. In all other experiments, extracts were incubated until fully activated before samples were removed for assay. All values of dopa oxidase activity are reproducible to within 10 per cent and the values cited are mean values of successive determinations from different homogenates of the designated strain of flies.

Results.—Differences in activation rates and dopa oxidase activity among laboratory strains: Dopa oxidase is extracted from fly material in an inactive form complexed with another protein called "the activator." Following a lag period, the length of which is controlled in part by the concentration of initial free activator, activation proceeds by an autocatalytic process involving separation of activator and proen-

zyme, the rate of which is a measure of the ratio of concentrations of activator and proenzyme.[1, 5, 6]

Activation rates and activity were measured in a number of Drosophila wild type and mutant strains to determine (1) the ranges of activation rates and activity, (2) the possibility of demonstrating a modal level of enzymatic activity, which would be indicative of an adaptive value, (3) any correlation between level of enzyme activity and body color, since this enzyme plays a role in melanin formation, and (4) the presence of discrete biochemical mutants. The results shown in Table 1

TABLE 1

COMPARISON OF DOPA OXIDASE SYSTEMS IN THIRTY-FIVE LABORATORY STRAINS OF *Drosophila melanogaster*

Genotype	Max. act. (D.U.)*	Lag period (min.)	$t^1/_2$ (min.)	Genotype	Max. act. (D.U.)	Lag period (min.)	$t^1/_2$ (min.)
Wild type strains:				Eye color mutants:			
Canton-S	80	15	75	p^p	25	50	75
Oregon-RC	353	45	140	*bw*	50	75	84
Oregon-R	500	30	190	*v*†	72	38	80
Oregon-S	870	20	155	*v bw*	175	30	85
Samarkand	960	20	155	g^3	300	40	140
Body color mutants:				w^a§	360	20	70
v s	144	55	80	*Cy/Pm*	375	30	104
$\widehat{yf}/Y$	280	80	110	*h st*	415	30	155
h th st p^p *cu sr* e^s	320	45	102	*g*	420	110	165
w s	400	35	102	$w^{\ddagger}$	425	30	240
s	490	40	120	*cl*	500	20	185
b	540	40	105	w^e	570	25	165
su^2-s w^a *cv t*	850	100	196	g^{50e}	600	85	158
				w^a‡	630	18	158
Other strains:							
L	360	45	140	*v*‡	720	20	136
$\widehat{XX}/Y$	520	50	135	*cm*	725	35	145
su^2s	540	40	95	*w*§	880	60	180
spl	640	40	98	g^{3d}	950	60	128
fa^N	725	20	130				

* One D.U. is the activity at 25°C which results in an increase of 0.001 O.D. units per minute at 480 mμ in a spectrophotometer, Beckman model B, when extracts are prepared in 0.067 *M* phosphate buffer, pH 7.0, 50 mg flies per ml buffer, and substrate is 20 μM dopa per ml.
† Derived from a stock maintained by the Genetics Division, University of California, Davis.
‡ Derived from a stock maintained by the Biology Department, Amherst College.
§ Derived from a stock maintained by the Zoology Department, University of California, Berkeley.

reveal that the rates of activation and the maximum activities vary over a wide range. These data are from extracts prepared from female flies.[7] The array of lag periods and the distribution of times of half-maximum activity reflect differences in the concentrations of initial unbound activator and proenzyme in the different strains. Since there is no correlation between maximum activity and the time of half-maximum activity or length of lag period, independent synthesis of the activator and proenzyme is inferred. Differences between extracts in inhibitor content affecting either the activation process or the activity of the enzyme were sought by mixing extracts both before and after activation, but in none of the cases tested were differences in degree of inhibition detected.

The values characterizing the dopa oxidase systems are inherited; thus, it is possible to investigate the genetic variables controlling these systems. It is reasonable to assume from the sample of strains surveyed that level of enzymatic activity and length of lag period are continuously as well as independently distributed. Since a large modal class has not been found, it is concluded that there are no obvious

indications that any specific level of enzymatic activity is advantageous to flies raised under laboratory conditions. There is no correlation between body color and the level of dopa oxidase activity in extracts.

Interaction of genes controlling enzyme activity: To explore the possibility that the wide divergencies in *in vitro* activity are due to single gene differences, crosses were made in preliminary experiments between strains with marked differences. Since the wild type designations and mutant symbols used to designate the strains in Table 1 bear no relationship to the dopa oxidase systems, these strains will henceforth be designated as *hi-* or *lo-* according to their maximum enzyme activities. The *lo-1*, *lo-2*, *hi-1* and *hi-2* strains are Canton-S, p^{p}, g^{50e} and Samarkand strains respectively. The strains used in the crosses are not the identical strains used in the survey but are derived from them. The results of the crosses shown in Table 2

TABLE 2

DOPA OXIDASE ACTIVITY IN HIGH AND LOW ACTIVITY STRAINS AND IN PROGENY OF CROSSES BETWEEN THEM

	Cross ♀ × ♂	Maximum Activity Observed (D.U.)	Maximum Activity Expected (D.U.) Geometric mean	Maximum Activity Expected (D.U.) Arithmetic mean
1.	*hi-1* × *hi-1*	650	...	...
2.	*hi-2* × *hi-2*	457	...	...
3.	*lo-1* × *lo-1*	64	...	...
4.	*lo-2* × *lo-2*	70	...	...
5.	*hi-1* × *lo-1*	150	204	357
6.	*hi-2* × *lo-2*	345	179	263
7.	*hi-2* × *lo-1*	94	171	260
8.	*lo-1* × *lo-2*	100	67	67
	lo-1:*lo-2* mixture*	65	67	67

* Extracts prepared using equal weights of *lo-1* and *lo-2* flies.

do not suggest that the differences in dopa oxidase activity in the parental strains are due to the existence of single gene differences. The values of the F_1's in relation to parental values show widely different relationships, and in no cases correspond to those expected from complete dominance or from simple additive or geometric effects of a single pair of alleles. The activities in progeny of crosses 5 and 7 are both less than the geometric mean of the parental activities, indicating the probable similarity in the manner of interaction of the genomes of *hi-1* and *hi-2* with *lo-1*. On the other hand, activity in progeny of cross 6 is much greater than the geometric mean of the parents. Comparison of crosses 6 and 7 shows that the genes of the two low-activity strains interact differently with the genes of the *hi-2* strain, indicating the possible presence of two different mechanisms controlling dopa oxidase activity. Heterozygotes of the two low-activity strains, cross 8, have more activity than either of the parental strains. This may be due to modification of the action of the genes from one of the low strains by genes from the other low strain. Another explanation is that the genomes of each low strain act independently in the heterozygote, one making available molecular entities which interact with those made available by the genes of the other strain. However, extracts prepared using equal amounts of the two low-activity strains as shown in the last row of Table 2 show additive activity, precluding the explanation of complementary stable molecular entities. Since these experiments were preliminary, final interpretations are withheld until information concerning the extent of intra-strain heterogeneity is presented.

Intra-strain selection for high and low enzyme activity: Lines with minimal heterogeneity were obtained by the method of selection and inbreeding. A series of single-pair matings from the *hi-2* strain were made and the progeny of each pair were assayed for activity. The pair whose progeny had the highest activity was the forerunner of a selected high line, *hi-3*. Progeny from this pair were mated in single pairs, from whose offspring the next generation of high-activity parents was selected. In a similar manner, a low-activity line, *lo-3* was simultaneously selected from the *hi-2* strain. A similar program of selection for high and low activity was attempted with the *lo-2* strain, but after three generations, no gains were made in either direction, and the lo-2 strain was regarded as operationally homogeneous with respect to the dopa oxidase system. The response to selection during the establishment of the *hi-3* and *lo-3* lines is shown in Figure 1. The high- and low-activity lines were

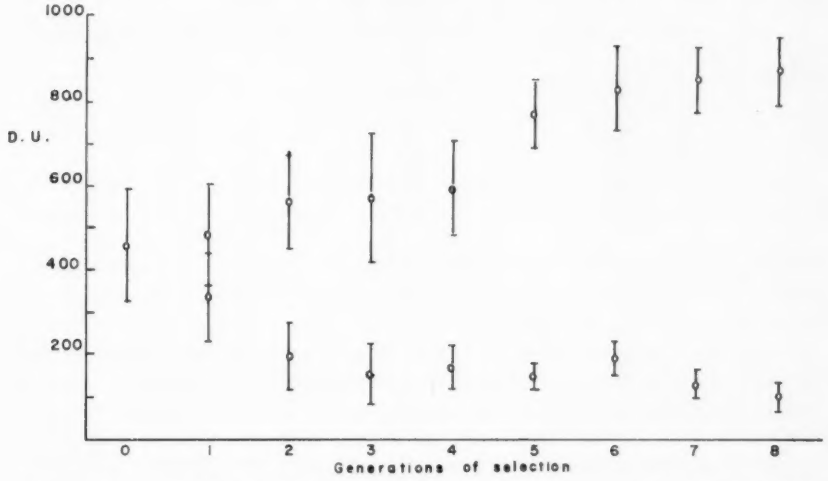

FIG. 1.—Response of the hi-2 strain to selection for high and low dopa oxidase activity during establishment of the *hi-3* and *lo-3* lines. Each circle represents the mean dopa oxidase value of extracts from the progeny of approximately 22 single-pair matings, the vertical lines indicate the standard deviations.

separated into two distinct populations after two generations.[8] No gains were made by selection beyond the eighth generation.

Analysis of strain differences: The three strains relatively homogeneous within themselves with respect to dopa oxidase activity (*lo-2*, *lo-3*, and *hi-3*) make it possible to investigate with greater precision genetic variables involved in control of level of activity. *In vitro* mixtures of these three lines show that the differences in activity are not due to the presence of extractable inhibitors in the low-activity strains. The results of crosses presented in Table 3 show that two distinct genetic mechanisms are operative in *lo-2* and *lo-3*. Activity in progeny of crosses 12 and 13 is less than the geometric mean between that of the parents, whereas activity in progeny of crosses 14 and 15 is significantly higher than the geometric mean of the parental activities. Comparison of these results with those recorded in Table 2 shows that the *lo-1* and *lo-3* genomes are similar to one another but that both

differ from the *lo-2* genome in the manner in which they interact with genes from the high-activity strains. In both experiments, crosses involving *lo-2* parents give rise to heterozygotes whose activity is much higher than that expected on the basis of comparison with all other crosses. Furthermore, in crosses of *lo-2* with the selected lines, activity in the heterozygotes is in the order of magnitude of that of the non-*lo-2* parent, suggesting the possibility that *lo-2* is homozygous for a gene or genes recessive to the alleles present in the selected lines. Heterozygotes of the two low-activity strains, crosses 16 and 17, have greater activity than either of the parental strains, similar to the result noted in the preliminary experiments (cross 8, Table 2). The data presented in Table 3 also show that there are reciprocal dif-

TABLE 3

DOPA OXIDASE ACTIVITY IN HOMOGENEOUS HIGH AND LOW ACTIVITY STRAINS AND IN THEIR HETEROZYGOTES

	Cross ♀ × ♂	Maximum Activity Observed (D.U.)	Maximum Activity Expected (D.U.) Geometric mean	Maximum Activity Expected (D.U.) Arithmetic mean
9.	*hi-3* × *hi-3*	850	...	...
10.	*lo-3* × *lo-3*	250	...	...
11.	*lo-2* × *lo-2*	70	...	...
12.	*hi-3* × *lo-3*	350	461	550
13.	*lo-3* × *hi-3*	370	461	550
14.	*hi-3* × *lo-2*	720	244	460
15.	*lo-2* × *hi-3*	850	244	460
16.	*lo-3* × *lo-2*	310	132	160
17.	*lo-2* × *lo-3*	410	132	160

ferences in crosses where *lo-2* is a parental strain. Heterozygotes whose mothers are from the *lo-2* strain have more dopa oxidase activity than do heterozygotes whose fathers are from the *lo-2* strain.[9]

To test the hypothesis that *lo-2* is homozygous recessive at a locus or loci with an easily recognized effect on dopa oxidase activity, the appropriate crosses were made. Assuming tentatively that the level of enzyme activity characteristic of the *lo-2* strain is due to a single locus and assigning the symbol α to designate this locus, homozygous α and heterozygous α individuals are expected in equal frequencies in a testcross. Among the progeny of a series of single-pair matings between the testcross progeny and the *lo-2* strain, two populations are expected: in one, all individuals should be homozygous; in the other, half the individuals should be homozygous and half should be heterozygous for *alpha*. The results in Table 4A show that in crosses between *lo-2* and *hi-3*, the progeny-tested testcross population falls into two distinct classes: one has the low activity characteristic of *lo-2* and the second has a level of activity intermediate between that of the *lo-2* and *hi-3* strains. The progeny-test data presented in Table 4A are in reality the values of a second backcross generation (because dopa oxidase activity was measured in the progeny produced by the first backcross generation crossed with the *lo-2* strain); therefore, in the progeny assayed, $^1/_8$ of the genes are derived from the *hi-3* strain and $^7/_8$ of the genes are from the *lo-2* strain. If the effects attributed to the assumed *alpha* locus are really due to two or more segregating loci, the probability is small that only the low value of 70 D. U. and the intermediate value of 460 D. U. would be observed in a 1:1 ratio in the progeny tests of the testcross population. The small variance observed further supports the interpretation of a single segregating locus. Although the possibility that the results are due to two

TABLE 4

DEMONSTRATION OF SECOND CHROMOSOME FACTORS CONTROLLING DOPA OXIDASE ACTIVITY

*A. Demonstration of recessive factor alpha in lo-2 strain**

Parents	850 ± 90 *hi-3* (+/+)	× 70 ± 11 *lo-2* (α/α)
Testcross	850 ± 80 F_1 (+/α)	× 70 ± 11 *lo-2* (α/α)
Progeny test of TC individuals†	460 ± 60 (+/α:α/α)	: 70 ± 11 (α/α)

* The numbers are the mean to the nearest 10 and standard deviation dopa oxidase units. Symbols in parentheses are the assumed genotype of each class.
† 159 test cross individuals were progeny tested: 79 were in the 460 D.U. class and 80 in the 70 D.U. class.

*B. Demonstration that alpha is located on the second chromosome**

Parents Cy/Pm; D/Sb (475) × *lo-2* (70)

F_1 $\frac{+\ \text{Cy}\ +}{\alpha + +}$ (135) × $\frac{+ +\ \text{Pm}}{\alpha + +}$ (430)

F_2† $\frac{+\ \text{Cy}\ +}{+ +\ \text{Pm}}$ (460): $\frac{+\ \text{Cy}\ +}{\alpha + +}$ (135): $\frac{+ +\ \text{Pm}}{\alpha + +}$ (470): $\frac{\alpha + +}{\alpha + +}$ (70)

* The numbers in parentheses are the maximum activities in D.U. The *alpha* symbol has been placed to the left of *Cy* arbitrarily. Preliminary mapping experiments show the *Alpha* locus to be near the right end of the left arm of chromosome II.
† The marked third chromosomes (D/+, Sb/+, D/Sb and +/+) are not shown but are randomly associated with the F_2 classes.

or more closely linked genes has not been ruled out, it is concluded as most likely that the *lo-2* strain is homozygous recessive at a locus determining dopa oxidase activity.

To determine the chromosome on which *alpha* is located, homozygous *alpha* individuals were crossed with a balanced-marker strain, and the F_2 classes were assayed for dopa oxidase activity. The pertinent results, shown in Table 4B, demonstrate that *alpha* is on the second chromosome since the low activity characteristic of homozygous *alpha* is always associated with the non-marked second chromosome. There are no indications that the low activity is associated with a sex-linked factor or any combination of the marked and non-marked third chromosomes. This cross further shows that there are two other similar second chromosome factors which control dopa oxidase activity. One factor is associated with the second chromosome held intact by the *Cy* inversion, and the other is associated with the second chromosome marked by the *Pm* inversion. Although there is no evidence that these factors are allelic, if we tentatively assume a multiple allelic series and designate the alleles in the *lo-2* strain, *Cy* chromosome, and *Pm* chromosome α^1, α^2, and α^3 respectively, the results could be explained by assuming that α^2 is dominant over α^1 and that both are recessive to α^3. Selection experiments with the balanced-chromosome strain, *Cy*/*Pm*, have shown that the modifying factors separated by selection are not on the second chromosome.

Discussion.—The discovery that the level of dopa oxidase activity is controlled by genetic factors which can readily be followed during segregation as well as by genetic variables which can be demonstrated by a selection program involving single-pair brother-sister matings indicates a complexity involving perhaps two determining systems at the molecular level. An hypothesis compatible with the data is that the *alpha* locus is a factor controlling dopa oxidase *synthesis*, while genes in other chromosomes and the cellular environment are factors regulating the level of enzymatic *activity* attained.[10] The term "controlling" is used to refer

to factors which primarily determine qualitative aspects of the dopa oxidase molecule, and the term "regulating" refers to influences which determine quantitative aspects of enzyme activity. This hypothesis does not imply that the *alpha* locus is the only controlling locus in the genome.

In the *lo-2* strain, homozygous *alpha* could exert a limiting influence on dopa oxidase activity, so that the regulating factors have no effect. This would account for the lack of variability in this strain. That *lo-2* contains genes that can modify the level of dopa oxidase activity seems probable from the results of the crosses of *lo-2* with other low-activity strains where the heterozygote has more activity than the parents. Since mixing experiments do not support the interpretation of molecular complementation, the results indicate that *lo-2* has probably contributed modifiers to the heterozygote which determine increased enzyme activity. Furthermore, in every case where homozygous *alpha* is placed in genetic backgrounds other than the *lo-2* strain, there is an accompanying lack of variability. In *lo-1*, the level of dopa oxidase activity is of the same order of magnitude as that in *lo-2*. Experience with *lo-1*, however, indicates the presence of intra-strain variability, though less of it than in other unselected lines. In the *lo-3* line, derived by selection, the level of dopa oxidase activity is greater than that in the other low-activity lines investigated. Although there is no proof that selection operated on quantitative and not qualitative aspects of the enzyme system during the establishment of the *hi-3* and *lo-3* lines, experiments testing the heat stability and substrate profiles of the enzyme from the two lines do not indicate qualitative differences. Presumably, therefore, most of the controlling factors in the *lo-3* line are the same as in the *hi-3* line, and the two strains differ only in their respective regulating genes.

Of great interest in this investigation is the observation that the *alpha* locus may be different in each of the three cases where it has been tested. Not enough chromosomes have been tested yet to warrant meaningful conclusions, but the possibility exists that there is a large number of alleles at this locus.

Most of the progress that has been made in recent years in our understanding of the genetic control of enzyme activity is due primarily to experiments with microorganisms. Although it is tempting to believe that the emerging concept of the genetic control of enzyme activity is similar for all enzymes and in all living systems, evidence for such generalizations is lacking, and it is therefore of interest to relate the present study to enzyme systems studied in microorganisms. It must be noted that the comparisons are made between microbial studies which are based on purified enzyme preparations where protein *synthesis* has been measured, while this Drosophila study is based on enzymatic *activity* in crude extracts. In bacteria, enzymatic activity has in some cases been shown to be determined by interactions at a secondary level rather than by the primary action of a single gene,[2, 3] and it is possible that in higher levels of organization, like Drosophila, interdependencies of a higher order of reactivity may be involved in determining enzyme activity. Although it is not possible at this time to determine the number of genes in Drosophila that control dopa oxidase synthesis and regulate its activity, a minimum number can be estimated. The present study, except for some data on the activation process of dopa oxidase presented with the survey of laboratory strains, is confined to measurement of maximum activity, which is a measure of the amount of proenzyme produced. There is reason to believe that

systems of genes exist which control and regulate the activity of the activator and also that there are genes that control and regulate an enzyme that activates the activator. Demonstration of these in the Drosophila genome would be of significance from a genetic and evolutionary point of view. Excluding such genes, which have not yet been demonstrated experimentally, and focusing attention only upon the selection experiment, it is likely that the results are due to at least two modifying factors. Thus, there must be at least three genes in Drosophila involved in dopa oxidase activity; at least two are modifying factors and one is a controlling factor. The modifying factors may determine the level of enzymatic activity through some regulating mechanism such as induction or repression. The sex differences and the reciprocal cross differences may be due to cytoplasmic inducers or repressors. Within this framework, the genetic control of dopa oxidase in Drosophila is similar to that of tyrosinase in Neurospora,[4] where one locus was found which controls the structural aspects of the enzyme molecule and two other loci were discovered which play a regulative role in quantitative aspects of enzyme synthesis. As in Neurospora, so too in Drosophila, the genes which affect the same enzyme system are not closely associated on the genetic map. In contrast, in *E. coli* the *i*, *o*, *z*, and *y* loci, which control and regulate β-galactosidase, are very closely linked.[2, 3]

An important difference between the tyrosinase system in Neurospora and the dopa oxidase system in Drosophila is that in the former tyrosinase is found as an active enzyme while in the latter dopa oxidase is extractable only in a complexed zymogen form and must be activated by another enzyme. This suggests that in Drosophila the enzyme system may be more complex and may be subject to a more intricate genetic control. This would be of interest from an evolutionary point of view. As a general rule, the amount of DNA and presumably the number of genes per cell is not correlated with phylogenetic status. It is reasonable to explain the higher degree of complexity in highly differentiated animals as compared to bacteria or fungi by assuming a greater degree of interdependency of gene products. In other words, it can be speculated that the higher degree of organization encountered in highly differentiated animals is actuated through a higher degree of pleiotropy and a higher degree of multiple-factor inheritance. If these suggestions are valid, the observed differences between the specific enzyme systems being compared here in Drosophila and Neurospora may have come about by natural selection operating less on autonomously acting genes and more on networks of genes during the course of evolution. This is compatible with the view that mutation and selection are the principle means of maintaining genetic variability in bacteria and fungi, while at higher levels of organization, genetic recombination is the important method of maintaining variability and at the same time serves as a mechanism for preserving networks of genes in a population. Thus, it is plausible to assume that a character at the biochemical level can become influenced by a large number of genes during evolution just like multigenically determined traits at the morphological level.

Summary.—The genetic control of dopa oxidase activity in Drosophila has been found to be multigenic. The results of genetic experiments are compatible with the hypothesis that there is a controlling factor located on the second chromosome and that there are at least two other factors present in the genome which regulate the level of dopa oxidase activity.

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[8] In subsequent studies, a *Cy/Pm* strain responded similarly to selection.

[9] Reciprocal differences in crosses involving *lo-2* were first observed in these experiments and therefore are recorded here. Detailed analysis of this phenomenon will be published elsewhere.

[10] Experiments performed by Mr. S. Waltman in our laboratory indicate that the enzyme systems of the *lo-2* and *lo-3* strains differ qualitatively. Almost all of the enzyme in extracts of *lo-2* is heat-labile, while extracts from *lo-3* contain both heat-labile and heat-stable enzyme. The two strains differ also with respect to their catalytic effect on the oxidation of the substrates dopa and catechol.

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*INHIBITION BY PERIODATE OF MATING IN ESCHERICHIA COLI K-12**

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The oxidation of carbohydrates by the periodate ion is the basis of the well-known McManus-Hotchkiss histochemical procedure.[1] It also played an important role in the identification of the mucopolysaccharide receptors for the influenza virus on the surface of tissue cells.[2] In the course of a routine survey of reagents for their effect on conjugation in *Escherichia coli*,[3] it was noted that periodate inhibited the mating reaction at a concentration that left most of the cells viable. As will be shown in the present paper, this inhibition can be attributed to the alteration of a substance, presumably carbohydrate, specifically associated with the conjugal activity of *male* cells.

Materials and Methods.—The general methods and the media have been described previously.[4] The strains used, which are all derived from *E. coli K-12*, and their genetic markers are as follows:[5]

W 2324 Hfr_2, Th^-, S^r.
W 3064 F^-, T^-, L^-, Th^-, Lac^-_1, Gal^-_7, Xyl^-_1, Ara^-_1, Mal^-_1, V^r_λ, V^r_1, V^r_6, S^r.
W 3086 F^-, M^-, S^r.
W 6 F^+, M^-.
W 3876 F^-, ♀$_3$, Lp^s, Lac^-_{11D3}, Mal^-_5, S^r.
W 3776 Hfr_2, Lp^s, T^-, L^-, Lac^-_1, V^r_1, V^r_6.
W 3780 Hfr_2, Lp^s, M^-Az^rS.

The strains were routinely grown overnight at 37°C in Difco "penassay" broth on a rotator, and the organisms (which numbered about 5×10^8 per ml) were washed once and resuspended at 0°C in distilled water at twenty times their original density. For periodate treatment, 0.1 ml of such a cell suspension was added to 0.4 ml of sodium metaperiodate solution made up in minimal medium without glucose.[4] The mixture was incubated for 10 minutes at 37°C, and the residual periodate was then destroyed by adding 4.5 ml of penassay broth. The treated cells were then mated with cells of the opposite mating type in penassay broth. After 15 minutes at 37°C, suitable dilutions were plated onto appropriate selected media for the estimation of recombinants. Routinely, the transfer of T^+L^+ alleles from Hfr_2 male cells into $T^-L^-F^-$ cells was studied by plating on minimal medium containing streptomycin to kill the male parental cells and also containing thiamin. The mating time of 15 minutes at 37°C was adequate for this purpose. The plates were thoroughly rubbed with a glass spreader until they were dry. This procedure has been found adequate to break up mating pairs with these strains and to reduce the background of plate recombination.

When plate recombination was encouraged (Table 2), the minimal medium was enriched 1:10 with penassay broth.

TABLE 1

EFFECT OF SODIUM PERIODATE ON MATING ABILITY OF MALE AND FEMALE STRAINS OF *Escherichia coli*

	♂* × ♀*	♂ × ♀*	♂* × ♀	♂ × ♀
Viable bacteria per 10^{-6} ml ♂:	53	99	46	84
♀:	98	120	148	120
Recombinants per 10^{-3} ml	2	356	1	466

Strains: The male was W 2324 and the female W 3064. 0.1 ml of twentyfold concentrated cells was added to 0.4 ml minimal medium without glucose, without or with M/2000 $NaIO_4$ (indicated by *), then incubated for 10 minutes at 37°C. Each tube received 4.5 ml penassay broth, and equal volumes of these suspensions were mixed in various combinations and incubated at 37°C for 15 minutes to allow mating. The recombinants were counted by plating on minimal medium containing streptomycin and thiamin.

TABLE 2

EFFECT OF SODIUM PERIODATE ON PAIR FORMATION IN *Escherichia coli*

Per cent survival after treatment	Treated male 19	Untreated male (100)	Untreated male (Mixture plated immediately)*
	Recombinants on plating		
0.01 ml	16	2,000	40
0.001 ml	0	206	

Strains: The male was W 2324 and the female W 3064. The treatments were similar to the prescription of Table 1. However, the experiment compared male cells that *were* or *were not* exposed to periodate for 15 minutes before mating. The mixture was incubated at 37°C for ten minutes to allow mating pairs to form, then diluted in penassay broth by gentle pipetting; 0.1 ml samples of suitable dilutions were mixed with 5 ml of melted enriched minimal agar and poured on prepared plates of minimal agar. After 5 hours incubation of 37°C, a top layer of 5 ml of minimal agar containing 1,200 μg/ml of dihydrostreptomycin was poured on, the plates were incubated at 37°C for two days, and the large recombinant colonies were counted. Survival after periodate treatment was estimated by viable counts on EMB egar.

* To estimate background of recombinants from matings occurring on the plate without the benefit of earlier pairing.

Viable counts of the treated suspensions were made concurrently on *EMB* agar. The yield of recombinants at the cell densities employed here is proportional to the product of the concentrations of the viable male and female organisms[6]; hence, the expected effect of reduced viability was readily calculated. However, we do not rely on this calculation when the periodate is found to have killed more than 90 per cent of the organisms. The most useful concentration was M/2000 sodium

periodate since this usually reduced the number of matings to about 1 per cent of the normal without killing more than 50 per cent of the treated organisms. It should be noted that the toxicity of $NaIO_4$ was quite sensitive to the temperature and media employed.

Periodate and iodate were assayed as in reference 7. Formaldehyde was detected as aldehyde by the Schiff reaction.

Results.—Inactivation of male cells: E. coli has a considerable capacity for reducing periodate to iodate, and packed organisms will destroy about an equal volume of M/10 $NaIO_4$ in a few minutes. Both iodate and formaldehyde, which may also be formed in the reaction, were inactive at concentrations equivalent to the periodate levels used in tests for toxicity and conjugal inactivation. Periodate is active at 0°C and remains active when it is freshly added to penassay broth, but the concentration must then be increased to compensate for the rapid destruction.

An illustrative experiment is shown in Table 1. It is seen that periodate has killed less than half of the cells but that when male cells were treated before mating, the number of recombinants was greatly reduced. Treatment of female cells has no significant effect, so the periodate does not make female cells infertile.

Recovery of male cells: When periodate-treated male cells were incubated in penassay broth at 37°C, there was no recovery of their virility for 30 minutes; after this, the virility increased and returned to normal after $1^1/_2$ to 2 hours.

Inhibition of F transfer: Treatment with periodate also inhibited the capacity of F^+ male cells to transfer the F agent to F^- cells. Since F^+ cells have relatively low fertility, the transfer of F was studied with a testing system employing the female strains described by Richter.[8] The F^+ strain used as F donor (W 6) was streptomycin-sensitive. The F^- strain used as F recipient (W 3086) was streptomycin-resistant. After mixing donor and recipient in penassay for a suitable time, the mixture was plated at a dilution which yielded 50 to 500 colonies per plate onto complete *EMB* medium containing streptomycin. On this medium, only the recipient formed colonies. Next day these colonies were replica-plated onto selective media spread with 0.5 ml of a young broth culture of a $♀_3$ strain, e.g. W 3876, on *EM* lactose agar. F^+ transfers to cells of W 3086 are detected as colonies which infect the lawn of strain W 3876, converting this to F^+, $♂_3$, a genotype of very high fertility. The ♂ patch undergoes frequent recombination with adjacent F^- W 3086 to yield M^+ Lac^+ recombinants which grow out conspicuously.

The finding of Jacob and Wollman[9] of a minimum time of about 4 minutes for the contact transmission of the F agent was confirmed. Treatment with periodate of the F donor greatly reduced the amount of F transfer, while treatment of the F recipient had no such effect. Periodate-treated donor cells also failed to transmit the mutant F factors described by Adelberg, et al.[10] and Hirota.[11]

Perborate and persulfate were found to be weakly male-attenuating. Perborate is sometimes found to compete with periodate in chemical combination with carbohydrate. Sodium perborate (M/100) did not block the subsequent male-attenuation by periodate, but rather tended to potentiate it, without, however, increasing the lethal effect.

Other effects of periodate: The "male" mating types of *E. coli* so far described are in fact able to act as weak females. By mating two Hfr_2 male strains, W 3776 and W 3780, for only 15 minutes and selecting for M^+ T^+ L^+ recombinants, one can

study W 3780 as ♂ × W 3776 as ♀, $T^+ L^+$ and not M^+ genes being transferred by Hfr_2 cells in this short time.[12] In this system, periodate-attenuated W 3776 retained its female function.

If a mixture of *Hfr* male and F^- female cells is incubated for a few minutes to allow mating to commence, the orderly transfer of *Hfr* markers is not prevented by the subsequent addition of periodate. Therefore, the periodate does not prevent the continuance of the mating process in those cell pairs which had commenced to mate before periodate was added. This suggests that the action of periodate is to prevent the first step in conjugation, the formation of mating pairs. This possibility was further investigated by the estimation of plate recombinants as indicated in Table 2. In this experiment, those microcolonies derived from a mating pair would contain both strains and these two strains would be able to mate within the microcolony to give streptomycin-resistant prototrophic recombinants. The latter would grow into large colonies, unlike the parental types, and their number would be an indication of the number of mating pairs originally present in the plate. It was found that prior treatment of the male cells with periodate greatly decreased the number of recombinant colonies in this experiment as compared to appropriate controls, thus affording evidence that it had indeed prevented the initial pairing of cells.

The fact that periodate does not interfere with matings which have already commenced should enable one to use this technique to study the kinetics of gene-transfer uncomplicated by subsequent matings. Preliminary experiments showed little difference from matings done without periodate, however, and this suggests that the majority of possible matings in broth in any case take place within a few minutes.

Conclusion and Discussion.—The simplest interpretation of these experiments is that male cells, carrying the *F* particle either in the cytoplasm or on the chromosome, are endowed with a specific conjugal substance on their surface. This substance would then be a periodate-reactive carbohydrate. The oxidation of this carbohydrate would prevent the effective recognition of female cells and formation of conjugal pairs. However, pairs that have once formed are so united that fertilization continues despite the alteration of this specific male substance. This proposal finds some support in the recent discovery by Ørskov and Ørskov[13] of a specific antigen which is regularly associated with the male character in *E. coli*. Attempts to assay this sexual receptor by the blocking power of extracts of male cells on sexual interaction have been inconclusive and further study of this hypothetical substance must await a more suitable assay, perhaps a serological one. No specific substances have so far been found associated with female cells and may be difficult to demonstrate since even male cultures are endowed with some female functional capacity. A more appropriate comparison may perhaps contrast sexually fertile F^- strains with completely sterile F° strains as suggested by Baron *et al.*[14]

The analogy of the sexual receptor to the receptors for influenza virus has already been mentioned as the principal basis for having tested the activity of periodate. Even more striking has been the finding that the mating capacity of one mating type in the yeast *Hansenula wingei* is susceptible to periodate. In this system, unlike *E. coli*, a complementary protein has, furthermore, been demonstrated on the cells of the opposite mating type, this mating type being susceptible to trypsin.[15] A number of enzymes, including snail stomach cytase, trypsin, chymotrypsin,

ribonuclease, and deoxyribonuclease have repeatedly been tested for their effect on mating in *E. coli* without any selective action having been found. The specific sensitivity of male function to periodate therefore remains a solitary clue to the chemical mechanism of conjugation.

The periodate-sensitive structure of male *E. coli* may show some analogy to the acrosome of the animal spermatozoon.[16] At least, the acrosome is reported to contain mucoproteins, stainable with Schiff's reagent after periodate treatment, and is believed to react specifically with the surface materials of the ovum.

Summary.—Periodate, and to a lesser extent, persulfate and perborate, devirilize the male mating types of *E. coli* K-12. The devirilized cells can still act as females, however, and female mating types are not affected by periodate at the relevant concentrations. Devirilized cells recover their virility after about 2 hours in broth culture. The action of periodate appears to be to prevent the formation of mating pairs of cells, probably by altering the surface properties of male cells. The effect is consistent with an oxidation of glycol links in a polysaccharide on the surface of male cells. Treatment of F^+ cells with periodate also makes them unable to transmit the *F* agent by infection.

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*COMPARISON OF THEORETICAL WITH OBSERVED VALUES OF THE PERIODS OF FREE OSCILLATION OF THE EARTH**

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1. *Introduction.*—When Benioff[1] announced in 1954 that he had observed an oscillation of 57 minutes on the seismogram of the Kamchatka earthquake of 1952 and suggested that it might be the free oscillation of the earth, there was no valid objection that could be leveled against those theoreticians who chose to adopt a wait-and-see attitude. This isolated observation, made for the first time after a century of seismometry, did not seem a sufficiently compelling reason for undertaking the cumbersome task of solving the system of coupled elastic and gravitational equations which govern the free oscillations of the earth, especially if the latter is to be represented by a realistic model based on empirical geophysical data. The methods used in earlier calculations by Love[2] for a homogeneous model and by Hoskins[3] for an analytically represented model were clearly no match for the detailed information we now possess on the internal constitution of the earth as derived, primarily, from seismic data.

One relatively simple aspect of the problem, the purely radial oscillations and the torsional oscillations, was readily disposed of,[4-9] since in these modes the general sixth-order differential system reduces to a second-order ordinary differential equation. The simplification arises from the direct dependence of the gravitational perturbation on the displacement in the case of radial oscillations, and from the disappearance of gravitational effects altogether in the torsional oscillations. Before tackling the problem of spheroidal oscillations in its entirety, another dodge was attempted, namely, the use of the variational method.[4, 10, 11] Fortunately for the future progress of the subject, even the variational method proved to be extremely laborious when the complexities of the empirically determined constitution of the earth, as well as the effect of gravitational forces, were taken into account.

The final formulation of the problem of spheroidal oscillations was given[9] in terms of a system of six simultaneous first-order differential equations. This system is particularly suited for integration on an electronic computer by the Runge-Kutta method, and it was chosen so as to avoid the need for differentiating explicitly the empirically determined elastic constants $\lambda(r)$ and $\mu(r)$, and the density $\rho(r)$. The period of free oscillation in the basic spheroidal $n = 2$ mode came out around 53.5 minutes for several models,[9] agreeing within the experimental error with Benioff's observed value of 57 minutes.

As the years passed and no further registrations of the earth's free oscillations were reported, doubts began to arise as to the reality of the original observation in the Kamchatka earthquake of 1952, especially since the experimental techniques were continually improving. These improvements at the hands of Benioff, Ewing, and Press[12-14] have extended the observed spectrum of surface waves in seismograms up to periods of 10 minutes. The amplitude of a progressive wave of such a long period penetrates down to the center of the earth, and its analysis can be given directly by the theory of free oscillations. It is with this application to progressive

TABLE 1

PROPERTIES OF THE BULLEN B EARTH MODEL

C_P and C_S denote the compressional and shear velocities as functions of the depth $a - r$. ρ denotes the density, λ and μ the elastic constants.

$a - r$ (km)	ρ (gm/cm³)	C_P (km/sec)	C_S (km/sec)	μ (10^{11} dynes/cm²)	λ (10^{11} dynes/cm²)	g (cm/sec²)
0	2.76	6.10	3.54	3.45	3.37	982
33	2.82	6.10	3.54	3.52	3.45	985
33	3.32	7.75	4.35	6.28	7.38	985
80	3.36	7.89	4.42	6.56	7.77	986
80	3.87	7.89	4.42	7.56	8.95	986
100	...	7.95	4.45	...	...	...
200	3.94	8.26	4.60	8.34	10.21	985
300	...	8.58	4.76	...	...	...
400	4.06	8.93	4.94	9.90	12.55	983
413	...	8.97	4.96	...	...	...
600	4.18	10.25	5.66	13.39	17.13	980
800	4.30	11.00	6.13	16.16	19.71	978
1,000	4.41	11.42	6.36	17.84	21.84	976
1,200	4.52	11.71	6.50	19.10	23.79	976
1,400	4.63	11.99	6.62	20.29	25.98	976
1,600	4.74	12.26	6.73	21.47	28.31	978
1,800	4.84	12.53	6.83	22.58	30.83	982
2,000	4.94	12.79	6.93	23.72	33.36	987
2,200	5.03	13.03	7.02	24.79	35.82	997
2,400	5.13	13.27	7.12	26.01	38.32	1,010
2,600	5.22	13.50	7.21	27.14	40.86	1,029
2,700	5.27	13.57	7.26	27.74	41.57	1.042
2,800	...	13.64	7.30	...	...	...
2,898	...	13.64	7.30	...	...	...
2,898	...	8.10	...	...	...	...
2,900	5.57	...	...	29.68	44.26	1,069
2,900	9.74	...	...	...	63.94	1,069
3,000	9.90	8.22	...	...	66.89	1,041
3,200	10.20	8.47	...	...	73.18	1,006
3,400	10.47	8.76	...	...	80.34	960
3,600	10.72	9.04	...	...	87.61	913
3,800	10.95	9.28	...	...	94.30	865
4,000	11.16	9.51	...	...	100.93	815
4,200	11.36	9.70	...	...	106.89	765
4,400	11.54	9.88	...	...	112.65	718
4,600	11.71	10.06	...	...	118.51	670
4,800	11.87	10.25	...	...	124.71	628
4,982	12.00	10.44	...	...	130.79	599
5,121	15.00	9.47	...	...	134.52	563
5,121	15.00	11.16	...	...	186.82	563
5,700	...	11.26	...	...	...	...
6,371	17.90	11.31	...	...	228.97	0

waves in mind that we have determined[15] the periods of free oscillation up to order $n = 61$ for the two models of the earth designated as "Bullen B" and "Gutenberg" and described in Tables 1 and 2, respectively.

These results were brought before the meeting of the International Union of Geodesy and Geophysics at Helsinki in July, 1960, simultaneously with the announcements by Benioff, Press, and Smith and by Ness, Harrison, and Slichter, of their observation of the free oscillations of the earth on the records of the Chilean earthquake of May, 1960. Benioff, Press, and Smith derived the periods of free oscillation from a power spectrum analysis of the records made by strain and pendulum seismographs.[16] Ness, Harrison, and Slichter succeeded in recording the Chilean earthquake with their tidal gravimeter.[17] The seismic and gravimetric observed periods of free oscillations are compared with the theoretical values in Tables 3 and 4. A distinguishing characteristic of the gravimetric records is that

TABLE 2

PROPERTIES OF THE GUTENBERG EARTH MODEL

C_p and C_s denote the compressional and shear velocities as functions of the depth $a - r$. ρ denotes the density, λ and μ the elastic constants.

$a - r$ (km)	ρ (gm/cm³)	C_p (km/sec)	C_s (km/sec)	μ (10^{11} dynes/cm²)	λ (10^{11} dynes/cm²)	g (cm/sec²)
0	2.74	6.14	3.55	3.45	3.42	982
19	3.00	6.58	3.80	4.33	4.32	983
38	3.32	8.20	4.65	7.18	7.97	984
50	3.34	8.17	4.62	7.13	8.04	985
60	3.35	8.14	4.57	7.00	8.20	985
70	3.36	8.10	4.51	6.83	8.38	986
80	3.37	8.07	4.46	6.70	8.54	986
90	3.38	8.02	4.41	6.57	8.59	986
100	3.39	7.93	4.37	6.47	8.37	986
125	3.41	7.85	4.35	6.45	8.11	987
150	3.43	7.89	4.36	6.52	8.31	988
175	3.46	7.98	4.38	6.64	8.76	989
200	3.48	8.10	4.42	6.80	9.23	989
225	3.50	8.21	4.46	6.96	9.67	990
250	3.53	8.38	4.54	7.28	10.24	991
300	3.58	8.62	4.68	7.84	10.92	992
350	3.62	8.87	4.85	8.52	11.45	993
400	3.69	9.15	5.04	9.37	12.15	995
450	3.82	9.45	5.21	10.37	13.38	996
500	4.01	9.88	5.45	11.91	15.32	997
600	4.21	10.30	5.76	13.97	16.73	998
700	4.40	10.71	6.03	16.00	18.47	998
800	4.56	11.10	6.23	17.70	20.79	997
900	4.63	11.35	6.32	18.49	22.66	995
1,000	4.74	11.60	6.42	19.54	24.71	993
1,200	4.85	11.93	6.55	20.81	27.41	990
1,400	4.96	12.17	6.69	22.20	29.06	986
1,600	5.07	12.43	6.80	23.44	31.45	983
1,800	5.19	12.67	6.90	24.71	33.90	982
2,000	5.29	12.90	6.97	25.70	36.63	981
2,200	5.39	13.10	7.05	26.79	38.92	984
2,400	5.49	13.32	7.15	28.07	41.27	989
2,600	5.59	13.59	7.23	29.22	44.80	997
2,800	5.69	13.70	7.20	29.50	47.80	1,011
2,898	9.40	8.10	...	...	61.71	1,037
3,000	9.55	8.23	...	...	64.72	1,015
3,500	10.15	8.90	...	...	80.42	908
4,000	10.7	9.50	...	...	96.58	800
4,500	11.2	9.97	...	...	111.39	631
4,982	11.5	10.44	...	...	125.34	469
5,121	12	10.75	...	...	138.66	422
6,371	12.3	11.31	...	...	157.34	0

they should not register torsional oscillations, since in the latter there is neither dilatation nor a radial component of displacement. This was found to be the case, as is shown in Table 4, where the observed torsional oscillations are only of seismic origin. Some observed *overtone* oscillations are compared with theory in Table 5.

2. *Discussion of Results.*—It is seen from Table 3 that for $n < 34$ the theoretical periods of spheroidal oscillations for model Bullen B are somewhat higher than the corresponding values for the Gutenberg model, while the reverse is true for $n > 34$. In the range $2 < n < 8$, the theoretical values for the two models differ by no more than the deviation of the seismic from the gravimetric observed values, which is less than one per cent. The same is true for $n > 22$. In the range $8 < n < 22$, on the other hand, the observed values are in distinctly better agreement with the Gutenberg than with the Bullen B values. The two sets of theoretical torsional periods and the observed seismic values shown in Table 4 are in mutual agreement

TABLE 3

COMPARISON OF THE THEORETICAL PERIODS OF FREE SPHEROIDAL OSCILLATION IN THE FUNDAMENTAL MODE FOR MODELS BULLEN B AND GUTENBERG WITH THE VALUES OBSERVED SEISMICALLY AND GRAVIMETRICALLY

	Theoretical		Observed			Theoretical		Observed	
n	Bullen B (min)	Gutenberg (min)	Seismic (min)	Gravimetric (min)	n	Bullen B (min)	Gutenberg (min)	Seismic (min)	Gravimetric (min)
0	20.65	...	...	20.46	31	4.26	4.24	4.27	4.270
2	53.70	53.52	54.7; 53.1	54.98; 52.80	32	4.16	4.15	4.18	4.167
3	35.50	35.33	35.9; 35.2	35.87; 35.24	33	4.06	4.06	4.08	4.089
4	25.73	25.54	25.8	25.85	34	3.97	3.97	4.00	3.999
5	19.85	19.66	19.8	19.83	35	3.88	3.89	3.92	3.920
6	16.12	15.92	16.0	16.07	36	3.79	3.81	3.82	3.837
7	13.64	13.44	13.5	13.42	37	3.71	3.73	3.73	3.755
8	11.95	11.74	11.81	11.78	38	3.63	3.66	3.66	3.681
9	10.77	10.54	10.56	10.57	39	3.56	3.59	...	3.612
10	9.88	9.65	9.66	9.685	40	3.49	3.52	...	3.475
11	9.18	8.95	8.98	8.934	41	3.42	3.45	...	3.405
12	8.61	8.38	8.37	8.368	42	3.35	3.39	...	...
13	8.11	7.90	7.88	7.882	43	3.29	3.33	...	...
14	7.68	7.48	7.47	7.468	44	3.23	3.27	...	...
15	7.30	7.11	7.10	7.101	45	3.17	3.22	...	...
16	6.96	6.78	6.78	6.780	46	3.11	3.16	...	...
17	6.66	6.49	6.48	6.488	47	3.06	3.11	...	...
18	6.39	6.23	6.23	6.232	48	3.00	3.06	...	...
19	6.14	6.00	6.01	6.002	49	2.95	3.01	...	...
20	5.91	5.78	5.78	5.778	50	2.90	2.96	...	...
21	5.70	5.59	5.59	5.608	51	2.85	2.91	...	...
22	5.48	5.40	5.39	5.423	52	2.81	2.87	...	...
23	5.29	5.24	5.26	5.255	53	2.76	2.82	...	...
24	5.12	5.08	5.10	5.104	54	2.72	2.78	...	...
25	4.96	4.94	4.96	4.959	55	2.68	2.74	...	...
26	4.82	4.81	4.83	4.828	56	2.64	2.70	...	...
27	4.70	4.68	4.69	4.703	57	2.60	2.66	...	...
28	4.58	4.56	4.59	4.585	58	2.56	2.62	...	...
29	4.47	4.45	4.47	4.476	59	2.52	2.58	...	...
30	4.37	4.34	4.37	4.366	60	2.48	2.55	...	...
..	...	...	...	...	61	2.45	2.51	...	...

to within one per cent. The $n = 2$ mode was recorded rather weakly. The mutual consistency of the theoretical and the observed overtone spheroidal oscillations shown in Table 5 is of the order of 2 per cent.

Altogether, the seismic observations comprise 37 fundamental spheroidal oscillations, 5 overtone spheroidal oscillations, and 10 fundamental torsional oscillations. The gravimetric observations comprise 41 fundamental spheroidal oscillations, 8 overtones, and, what is equally important, no torsional oscillations. The agreement of the theoretical with the observed values of the periods is in most cases better than one per cent, with a distinct preference shown for the Gutenberg (low-velocity) model. This attests to a considerable insight which we now possess about the internal constitution of the earth. The point is illustrated in the last column of Table 5, giving theoretical periods[9] for a simplified model α. It consists of a homogeneous mantle and a homogeneous core, each having densities and elastic constants equal to the corresponding mass-average values for model Bullen B. The periods for model α differ by as much as 5 per cent from the Bullen B and Gutenberg values.

3. *Rotational Splitting of the Free Oscillations of the Earth.*—Already at the Helsinki meeting of the IUGG, it was reported to Press and Slichter by cable dispatches that the $n = 2$ and $n = 3$ frequencies appear as doublets in both the seismic

TABLE 4

COMPARISON OF THE THEORETICAL PERIODS OF FREE TORSIONAL OSCILLATIONS FOR MODELS BULLEN B AND GUTENBERG WITH THE OBSERVED SEISMIC VALUES*

n	Bullen B (min)	Gutenberg (min)	Seismic (min)	n	Bullen B (min)	Gutenberg (min)	Seismic (min)
2	44.16	43.61	42.3	31	4.14	4.19	...
3	28.60	28.23	28.6	32	4.03	4.08	...
4	21.91	21.62	21.8	33	3.92	3.98	...
5	18.07	17.84	17.9	34	3.82	3.88	...
6	15.54	15.36	15.5	35	3.73	3.78	...
7	13.72	13.57	13.5	36	3.64	3.69	...
8	12.33	12.22	12.3	37	3.56	3.61	...
9	11.23	11.15	11.21	38	3.48	3.53	...
10	10.34	10.28	10.33	39	3.40	3.45	...
11	9.59	9.55	9.60	40	3.33	3.37	...
12	8.95	8.93	...	41	3.26	3.30	...
13	8.40	8.39	...	42	3.19	3.23	...
14	7.92	7.93	...	43	3.12	3.17	...
15	7.50	7.51	...	44	3.06	3.10	...
16	7.12	7.15	...	45	3.00	3.04	...
17	6.78	6.81	...	46	2.95	2.99	...
18	6.48	6.52	...	47	2.89	2.93	...
19	6.20	6.24	...	48	2.84	2.87	...
20	5.95	5.99	...	49	2.79	2.82	...
21	5.72	5.77	...	50	2.74	2.77	...
22	5.50	5.55	...	51	2.69	2.72	...
23	5.31	5.36	...	52	2.64	2.68	...
24	5.12	5.18	...	53	2.60	2.63	...
25	4.95	5.01	...	54	2.56	2.59	...
26	4.79	4.85	...	55	2.52	2.55	...
27	4.65	4.70	...	56	2.48	2.51	...
28	4.51	4.56	...	57	2.44	2.47	...
29	4.38	4.43	...	58	2.40	2.43	...
30	4.25	4.31	...	59	2.37	2.39	...
				60	2.33	2.35	...

* Between $n = 20$ and $n = 60$, the periods were computed at steps of 5 and interpolated at intermediate values.

and the gravimetric records of the Chilean earthquake of May, 1960, and it was then suggested[18] that this splitting is due to the rotation of the earth. The conjecture was made on the basis of the known classical result[19] that, in the case of a rotating

TABLE 5

COMPARISON OF THE THEORETICAL WITH THE OBSERVED PERIODS OF SPHEROIDAL OSCILLATION IN THE OVERTONES

		Theoretical		Observed		Theoretical
n		Bullen B (min)	Gutenberg (min)	Seismic (min)	Gravimetric (min)	α (min)
0	Fundamental	20.68	20.72	...	20.46	...
	Overtone I	10.01	10.00	...	10.21	...
2	Fundamental	53.70	53.52	54.7; 53.1	54.98; 52.80	56.0
	Overtone I	24.75	24.32	24.5	24.65	25.2
	Overtone II	15.49	15.15	15.1	15.03	16.3
	Overtone III	9.78	9.67	9.92	...	10.4
	Overtone IV	7.99	7.98	...	...	7.9
3	Fundamental	35.50	35.33	35.9; 35.2	35.87; 35.24	...
	Overtone I	17.94	17.63	17.6	17.88; 17.68	...
	Overtone II	13.58	13.25	...	13.53	...
4	Fundamental	25.73	25.54	25.8	25.85	...
	Overtone I	14.38	14.11	...	14.30	...
	Overtone II	12.25	11.96	...	12.08	...
5	Fundamental	19.85	19.66	19.8	19.83	...
	Overtone I	12.32	12.05	...	12.24	...
	Overtone II	11.12	10.92	11.01	...	...

circular basin, the waves advancing relative to the water in the direction of rotation have a longer period than the waves moving in the opposite direction. In order to obtain a qualitative check on this hypothesis, it will be instructive to ascertain the magnitude of the frequency split due to a slow rotation of a circular basin and by analogy to infer the order of magnitude of the frequency split that the rotation of the earth could be expected to introduce.

Following Lamb,[19] let ω denote the angular rotation of the basin, a its radius, and h its depth. With reference to a cylindrical system of coordinates (r,θ) rotating with the basin, the elevation of the free surface ξ is represented by

$$\xi = AJ_s(kr)e^{i(\sigma t + s\theta)}. \tag{1}$$

The periods of free oscillation are given by

$$xJ_s'(x) + (2s\omega/\sigma)J_s(x) = 0, \; x = ka, \tag{2}$$

where

$$\sigma^2 = 4\omega^2 + x^2gh/a^2. \tag{3}$$

For positive values of σ and ω, a wave advancing relative to the water in the direction of rotation has a negative value of s, and a wave advancing in the opposite direction has a positive value of s. It is clear from equation (2) that the roots x are different in the two cases, and therefore the frequencies given by (3) will be different in magnitude for the two types of waves.

Let

$$x = x_0 + \alpha, \tag{4}$$

where α is of the first order in the small quantity ω/σ. Then,

$$J_s'(x_0) = 0, \; \sigma_0 = (x_0/a)\sqrt{gh}, \tag{5}$$

and, on making use of the differential equation obeyed by the Bessel function $J_s(x)$, we get

$$\alpha = \frac{2s\omega x_0}{\sigma_0(x_0^2 - s^2)}. \tag{6}$$

To the first order in ω/σ we thus obtain from (3)

$$\sigma - \sigma_0 = \tau\omega, \tag{7}$$

where

$$\tau = \frac{2s}{(x_0^2 - s^2)}. \tag{8}$$

The values of τ for $s = 2$ and $s = 3$ are rather close, being 0.75 and 0.69 respectively. With σ_0 and ω positive, the frequency σ_1 of the wave advancing in the direction of rotation is negative, and is given by

$$\sigma_1 = \sigma_0 + \tau_+\omega, \tag{9}$$

while the frequency σ_2 of the wave traveling in the opposite direction is

$$\sigma_2 = \sigma_0 + \tau_+\omega, \tag{10}$$

$$\sigma_2 - \sigma_1 = 2\tau_+\omega. \tag{11}$$

Here τ_+ denotes the value of τ for positive s.

Since τ does not depend on the physical parameters of the system, which could enter through the nondimensional quantity $\beta = 4\omega^2a^2/gh$, we shall test whether the observed splitting of the frequencies of the free oscillations of the earth agree in order of magnitude with relation (11) if we substitute there for ω the value 7.27×10^{-5} sec^{-1} of the angular rotation of the earth.

TABLE 6

	Seismic			Gravimetric			
n	$10^3\sigma_1$	$10^3\sigma_2$	$\frac{\sigma_2 - \sigma_1}{\omega}$	$10^3\sigma_1$	$10^3\sigma_2$	$\frac{\sigma_2 - \sigma_1}{\omega}$	2τ
2	1.914	1.972	0.80	1.905	1.983	1.1	1.5
3	2.917	2.975	0.8	2.919	2.972	0.7	1.4

σ_1 and σ_2 denote the observed frequency doublets. τ denotes the values of $2s/(x_0^2 - s^2)$ for $s = 2$ and $s = 3$. $\omega = 7.27 \times 10^{-5}$ sec^{-1}.

In Table 6 are shown values of $(\sigma_2 - \sigma_1)/\omega$ for $n = 2$ and $n = 3$, where σ_2 and σ_1 denote the doublets of frequency of the free oscillations observed seismically and gravimetrically, as given in Table 3.

The observed values of the relative frequency splitting $(\sigma_1 - \sigma_2)/\omega$ agree within a factor of 2 with the relative frequency splitting 2τ of a circular basin rotating about an axis normal to its surface. Actually, in the case of the earth, the component of angular velocity normal to the surface varies from the full value ω at the pole to 0 at the equator, so that an effective angular velocity equal to about $\omega/2$ is not unreasonable. The order-of-magnitude argument presented here, based on the analogy with the rotating circular basin, seems, therefore, to support the hypothesis about the rotational origin of the observed frequency splitting of the free oscillations of the earth. It would be of interest to make a more accurate determination of the frequency splitting from the records and to study the present discrepancy between the gravimetric and seismic observations of the splitting at $n = 2$. A theoretical study of the rotational splitting of the free oscillations of the earth is under way.

4. *Summary.*—The theoretical values of the periods of free oscillation of the earth, in both the spheroidal and torsional modes, are compared with the experimental values derived by Benioff, Press, and Smith from the seismograms (52 periods) and by Ness, Harrison, and Slichter from the gravimetric records (49 periods) of the Chilean earthquake of May, 1960. Two models of the earth are considered: model "Bullen B" and the "Gutenberg" model, the latter being characterized by the occurrence of a low-velocity layer at a depth of about 150 km. The periods deduced from power-spectra analyses of the seismic and gravimetric records are in mutual agreement to within one per cent. The theoretical periods of spheroidal oscillations for the Gutenberg model agree with the observed values to within one per cent, and this is true also for model Bullen B at periods less than 5 minutes. In the period range of 5 to 11 minutes, the observed values are systematically lower by about 2 per cent than the Bullen B values, thus favoring the Gutenberg model.

As expected on theoretical grounds, no torsional oscillations were found in the spectrum of the gravimetric records. The torsional oscillations recorded on the

seismograms agree in period to within one per cent with both the Bullen B and Gutenberg values, except for the $n = 2$ mode, which was, however, recorded weakly. An order-of-magnitude argument is presented in support of the hypothesis of the rotational origin of the frequency splitting of the free oscillations of the earth.

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RINGS AND QUASICONFORMAL MAPPINGS IN SPACE

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I will describe in this paper a number of new results on rings and quasiconformal mappings in space. Complete proofs for all of this work will appear shortly. For the sake of simplicity, I will restrict the discussion to a 3-space, though it is clear how many results carry over to an n-space.

RINGS IN SPACE

1. *Conformal Capacity.*—A ring is defined as a domain whose complement consists of two components, one of which is unbounded. Given a ring R, we let C_0 and C_1 denote, respectively, the bounded and unbounded components of $\mathcal{C}R$. We

further let $B_0 = \partial C_0$ and $B_1 = \partial C_1$. These are the components of ∂R. (Given any set E, we let $\mathcal{C}E$ denote its complement and ∂E its boundary.)

Next let u be any function which is continuously differentiable in R and has boundary values 0 on B_0 and 1 on B_1. Following Loewner,[11] we define the *conformal capacity* of R as

$$\Gamma(R) = \inf_u \int_R |\nabla u|^3 d\omega, \tag{1}$$

where the infimum is taken over all such functions u.

As in the case of the plane, it is often convenient to work with a slightly larger class of competing functions u. We say that a function u is ACL, or *absolutely continuous on lines*, in a domain D if, given any sphere U, $\bar{U} \subset D$, u is absolutely continuous on almost all line segments in U which are parallel to the coordinate axes. If u is continuous and ACL in a ring R, then u has partial derivatives a.e. in R. If, in addition, u has boundary values 0 on B_0 and 1 on B_1, it can be shown that

$$\Gamma(R) \leqslant \int_R |\nabla u|^3 d\omega. \tag{2}$$

We call such a function u an *admissible function* for the ring R. It then follows from (2) that the infimum in (1) may be taken over the class of admissible functions u.

We can also define conformal capacity by means of extremal lengths. For example, let ρ be nonnegative and continuous in R and let

$$L(\rho) = \inf_\gamma \int_\gamma \rho \, ds \tag{3}$$

where γ is any locally rectifiable curve in R joining B_0 and B_1, and let

$$V(\rho) = \int_R \rho^3 d\omega. \tag{4}$$

Then, it is easy to show that

$$\Gamma(R) = \inf_\rho \frac{V(\rho)}{L(\rho)^3},$$

where the infimum is taken over all ρ for which $L(\rho)$ and $V(\rho)$ are not simultaneously 0 or ∞.

2. *Modulus of a Ring.*—The modulus of a ring is now defined in terms of the conformal capacity as follows:

$$\operatorname{mod} R = \left(\frac{4\pi}{\Gamma(R)}\right)^{1/2}. \tag{5}$$

This modulus behaves in many ways like the familiar modulus of a plane ring, usually defined by means of conformal mapping. For example, if R is bounded by concentric spheres of radii a and b, $a < b$, then,

$$\operatorname{mod} R = \log \frac{b}{a}. \tag{6}$$

Or if R' and R'' are disjoint rings each of which separates the boundary components of R, then,

$$\operatorname{mod} R \geqslant \operatorname{mod} R' + \operatorname{mod} R''. \tag{7}$$

A sequence of sets $\{E_n\}$ is said to *converge uniformly* to a set E if, given any $\epsilon > 0$, there exists an N such that $n > N$ implies that each point of E_n lies within distance ϵ of E and that each point of E lies within distance ϵ of E_n.

The function mod R is continuous in the following sense. Let $\{R_n\}$ be a sequence of rings and R a bounded ring. If each component of ∂R_n converges uniformly to the corresponding component of ∂R, then,

$$\operatorname{mod} R = \lim_{n \to \infty} \operatorname{mod} R_n. \tag{8}$$

An important theorem due to Loewner[11] shows that the conformal capacity of a ring is positive if and only if both boundary components are nondegenerate. Hence, as in the case of the plane, the modulus is finite if and only if neither boundary component reduces to a point.

3. *Extremal Function.*—If R is a ring with nondegenerate boundary components, then there exists an extremal admissible function u for which

$$\Gamma(R) = \int_R |\nabla u|^3 d\omega. \tag{9}$$

The function u is unique, and we call it the *extremal function* for R. It is the space analogue for the harmonic measure whose Dirichlet integral yields the capacity for a plane ring. Loewner's theorem shows that no such u can exist when one of the boundary components is degenerate.

The extremal function u satisfies the variational condition

$$\int_R |\nabla u| \nabla u \cdot \nabla v d\omega = 0 \tag{10}$$

for each function v which is continuously differentiable and has compact support in R. Applying recent results of de Giorgi[6] and C. B. Morrey[16] to (10) we can establish the following result.

THEOREM 1. *If u is extremal for R and if constants m and M exist so that*

$$0 < m \leqslant |\nabla u| \leqslant M < \infty \tag{11}$$

a.e. in R, then u is real analytic and

$$\operatorname{div}(|\nabla u| \nabla u) = 0 \tag{12}$$

in R.

SYMMETRIZATION OF RINGS

4. *Spherical Symmetrization.*†—Given an open set G, we define a second open set G^* as follows. Let S be the spherical surface $|x| = r$, $r \geqslant 0$. Then $G^* \cap S$ is to be null if and only if $G \cap S$ is null. Next, $S \subset G^*$ if and only if $S \subset G$. For the remaining case, let S meet G in a set whose measure on S is A. Then, G^* is to meet S in the single open spherical cap of area A which has center on the negative half of the x_1-axis and does not meet the positive half of this axis.

Given a closed set F, we define the closed set F^* exactly as above except that in the last case we take the spherical cap to be closed.

Now let R be any ring. Then, $R \cup C_0$ is open, C_0 is closed, and we define the *spherical symmetrization* of R by means of the equation

$$R^* = (R \cup C_0)^* - C_0^*. \tag{13}$$

R^* is a ring for which we can establish the following fundamental extremal property.

THEOREM 2. *mod* $R \leq$ *mod* R^*.

5. *Point Symmetrization.*—Given an open set G with $mG < \infty$ we define G^{**} as the open sphere with center at the origin and volume mG. For a closed set, the sphere is taken to be closed. Then, given any ring R with $mR < \infty$, we define the *point symmetrization* of R as

$$R^{**} = (R \cup C_0)^{**} - C_0^{**}. \tag{14}$$

Again R^{**} is a ring, and we can prove the following space analogue of a theorem due to Carleman.[5]

THEOREM 3. *mod* $R \leq$ *mod* R^{**}.

6. *The Grötzsch and Teichmüller Rings.*—For $a > 1$, let $R_G = R_G(a)$ denote the ring bounded by $|x| = 1$ and the ray $a \leq x_1 < \infty$, $x_2 = x_3 = 0$. Next, for $b > 0$, let $R_T = R_T(b)$ denote the ring bounded by the segment $-1 \leq x_1 \leq 0$, $x_2 = x_3 = 0$ and the ray $b \leq x_1 < \infty$, $x_2 = x_3 = 0$. These are the space analogues of the plane rings studied by Grötzsch and Teichmüller.[22]

If we set

$$\text{mod } R_G = \log \Phi(a), \qquad \text{mod } R_T = \log \Psi(b), \tag{15}$$

then we can show that

$$\Psi(b) = \Phi(\sqrt{b+1})^2 \tag{16}$$

and that $\Phi(a)/a$ increases monotonely from 1 to a constant λ, $\lambda < 13$, as a increases from 1 to ∞. In particular,

$$a < \Phi(a) < \lambda a. \tag{17}$$

Now let R be any ring with the property that C_0 contains the origin and a point of $|x| = 1$ while C_1 contains a point of $|x| = b$. Then R^* separates the boundary components of R_T and we obtain

$$\text{mod } R \leq \log \lambda^2(b+1) \tag{18}$$

from Theorem 2 and from (16) and (17).

QUASICONFORMAL MAPPINGS IN SPACE

7. *Definitions.*—Let $y(x)$ be a topological mapping or homeomorphism of a domain D. For convenience, we introduce the following functions to measure various distortions of $y(x)$ at a point $x \in D$. First, we let

$$H(x) = \limsup_{r \to 0} \frac{L(x,r)}{l(x,r)}, \qquad I(x) = \limsup_{r \to 0} \frac{L(x,r)}{r}, \tag{19}$$

where for small $r > 0$,

$$L(x,r) = \max_{|x - x'| = r} |y(x) - y(x')|, \qquad l(x,r) = \min_{|x - x''| = r} |y(x) - y(x'')|. \tag{20}$$

Next, we let

$$J(x) = \limsup_{r \to 0} \frac{mU'}{mU}, \tag{21}$$

where U is the sphere with center x and radius r and where U' is the image of U under $y(x)$. (E' will denote the image of any set $E \subset D$ under $y(x)$.) At a point of differentiability, $I(x)$ is the maximum stretching, $H(x)$ is the ratio of the maximum to minimum stretching, and $J(x)$ is the absolute value of the Jacobian.

We adopt the following definition for quasiconformality.

DEFINITION.—*A topological mapping $y(x)$ of a domain D is said to be K-quasiconformal, $1 \leqslant K < \infty$, if*

$$\frac{1}{K} \bmod R \leqslant \bmod R' \leqslant K \bmod R \tag{22}$$

for all bounded rings R, $\bar{R} \subset D$. A quasiconformal mapping is one which is K-quasiconformal for some K.

Plane quasiconformal mappings can also be defined as above by means of rings. For a proof, see reference 9.

Let $y(x)$ be a continuously differentiable topological mapping with a nonvanishing Jacobian and assume that $y(x)$ maps infinitesimal spheres onto infinitesimal ellipsoids so that the ratio of the major to minor axes does not exceed K; that is, $H(x) \leqslant K$ everywhere in D. Then, $y(x)$ is K-quasiconformal by the above definition.

The converse is, of course, not true. However, we can show that a K-quasiconformal mapping is differentiable with $J(x) > 0$ a.e. in D and that

$$H(x) \leqslant \mu \tag{23}$$

at all points of D, where μ is a constant which depends only on K.

Equation (8) implies that the quasiconformal mappings of a domain D have the following compactness property. If $\{y_n(x)\}$ is a sequence of K-quasiconformal mappings which converge uniformly on compact subsets to a topological mapping $y(x)$, then $y(x)$ is also K-quasiconformal.

The following result allows us to formulate an alternative analytic definition for quasiconformality.

THEOREM 4. *Let $y(x)$ be a topological mapping of D. Then,*

$$\bmod R' \leqslant K \bmod R \tag{24}$$

for all bounded rings R, $\bar{R} \subset D$, if and only if each component of $y(x)$ is ACL and

$$I(x)^3 \leqslant K^2 J(x) \tag{25}$$

a.e. in D.

For plane analogues of this result, see references 2, 8, and 18.

8. *Boundary Correspondence.*—Let $y(x)$ be a K-quasiconformal mapping of $|x| < 1$ onto $|y| < 1$. Using Theorem 4 and arguing as in reference 14, we can extend $y(x)$ to a topological mapping of $|x| \leqslant 1$ onto $|y| \leqslant 1$. Then, if $y(0) = 0$, reflection yields a K-quasiconformal mapping of $|x| < \infty$ onto $|y| < \infty$.

Carrying this over to the case where $y(x)$ maps a half-space onto a half-space and applying (23) and Theorem 3 of reference 7, we obtain the following result.

THEOREM 5. *Each quasiconformal mapping of $x_1 > 0$ onto $y_1 > 0$ can be extended to a topological mapping of $x_1 \geqslant 0$ onto $y_1 \geqslant 0$. The induced boundary correspondence is itself a plane quasiconformal mapping of $x_1 = 0$ onto $y_1 = 0$.*

We note in particular that this boundary correspondence is absolutely continuous. An example due to Beurling and Ahlfors[3] shows this need not be true of the boundary correspondence induced by a quasiconformal mapping of a half-plane onto a half-plane.

9. *Distortion Theorems.*—Using Theorem 3 and an argument due to Morrey,[15] we can establish the following distortion theorem. Similar results are established in references 4 and 20.

THEOREM 6. *If $y(x)$ is a K-quasiconformal mapping of D onto D' and if $mD' < \infty$, then,*

$$|y(x) - y(x')| \leqslant A \left(\frac{|x - x'|}{d} \right)^{1/K} \tag{26}$$

whenever $|x - x'| \leqslant d/2$. Here d is the distance $x \epsilon D$ lies from ∂D and A is a constant which depends only on K and mD'.

Next, the results of the preceding section together with inequality (18) yield the following space analogue of a theorem due to Ahlfors[1] and Mori.[13]

THEOREM 7. *If $y(x)$ is a K-quasiconformal mapping of $|x| < 1$ onto $|y| < 1$ with $y(0) = 0$, then,*

$$|y(x) - y(x')| \leqslant A |x - x'|^{1/K} \tag{27}$$

for $|x|, |x'| < 1$. Here A is an absolute constant.

10. *Conformal Mappings.*—We conclude by describing the 1-quasiconformal mappings. Let $y(x)$ be a topological mapping of a domain D which is a Moebius transformation. (The Moebius transformations are the mappings of the extended space onto itself generated by similarity transformations and inversions.) From (1), it is clear that

$$\operatorname{mod} R' = \operatorname{mod} R \tag{28}$$

for all rings R, $\bar{R} \subset D$, and hence $y(x)$ is 1-quasiconformal. We shall now show that the converse is true.

THEOREM 8. *Let $y(x)$ be a topological mapping of D and let (28) hold for all bounded rings R, $\bar{R} \subset D$. Then $y(x)$ is a Moebius transformation.*

The proof is based upon the fact that such a mapping must preserve the extremal functions for rings. In particular, if R' is the image of a bounded ring R and if $v(y)$ is the extremal function for R', then (25) and (28) imply that $u(x) = v(y(x))$ is extremal for R.

Now fix $x' \epsilon D$ and pick R, $\bar{R} \subset D$, so that R' is bounded by concentric spheres with center at $y' = y(x')$. Then,

$$v(y) = A \log |y - y'| + B, \tag{29}$$

and, applying (26), we see that u satisfies the hypothesis (11) of Theorem 1. Hence, u is real analytic in R, and by varying x', we can show that the components of $y(x)$ are real analytic in D.

Next, (26) applied to the inverse mapping $x(y)$ shows that $y(x)$ has a nonvanishing Jacobian in D. By continuity,

$$I(x)^3 = J(x) \qquad \text{or} \qquad H(x) = 1 \tag{30}$$

everywhere in D, and we can finally appeal to a classical theorem of Liouville to conclude that $y(x)$ is a Moebius transformation. (See, for example, pp. 197–200 in ref. 10).

For some time, it has been an open question as to what differentiability hypotheses are necessary for Liouville's theorem to hold. A recent proof by Nevanlinna[17] asks that $y(x)$ be four times differentiable and have a nonvanishing Jacobian.

The following result shows that no such differentiability hypotheses are required. It is the space analogue of a theorem due to Menchoff.[12]

THEOREM 9. *Let $y(x)$ be a topological mapping of D and let $H(x) = 1$ everywhere in D. Then $y(x)$ is a Moebius transformation.*‡

The fact that $H(x) = 1$ everywhere in D implies that

$$\operatorname{mod} R' \leqslant \operatorname{mod} R \tag{31}$$

for all rings R, $\bar{R} \subset D$. Next, we can show for $x(y)$ that either $H(y) = 1$ or $I(y) = 0$ at each point of D'. This implies that

$$\operatorname{mod} R \leq \operatorname{mod} R' \tag{32}$$

for all rings R', $R' \subset D'$, and the desired conclusion follows from Theorem 8.

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† See pp. 205–210 in ref. 19.

‡ While this manuscript was in preparation, a paper by Rešetnyak[21] appeared in which essentially the same theorem is announced.

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*LOCALLY UNKNOTTED COMBINATORIAL $n-1$ MANIFOLDS IN E^n HAVE SHELL NEIGHBORHOODS**

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1. *Introduction.*—In a Euclidean 3-space, E^3, a polygonal simple closed curve may be embedded in several ways, i.e., there are distinct knot-types. Similarly, a polyhedral 2-manifold may be embedded in E^3 in several ways if it is of positive genus. However, the polyhedral manifold of genus zero can be embedded in E^3 in only one way if orientation is ignored. The assertion that a polyhedral 2-sphere may be embedded in only one way is capable of at least two (equivalent, as it turns out) interpretations. First, if A is the bounded component of the complement of the 2-sphere M, then the closure of A is a closed 3-cell. Second, there is a semilinear homeomorphism on E^3 carrying the closure of A onto the closure of a tetrahedron.

For nonpolyhedral sets, the situation is less simple. L. Antoine gave an example, now classical, of a simple arc embedded in E^3 in such a way that its complement is not simply connected. Hence, its complement could not be homeomorphic to the complement of an ordinary interval and even less could the complement be semilinearly equivalent to that of an ordinary interval. It is easy to modify examples of this type to form 2-manifolds, even a topological 2-sphere, whose interior is not homeomorphic to that of a polyhedral prototype.

It is natural to inquire as to the nature of additional properties to be imposed so that the embeddings will be similar to those of a prototype. Probably the most general result that holds in E^3 is the result of Bing and Moise that asserts that locally tame sets are tame. Various results have been attained that concern the embeddings of 1-manifolds and 2-manifolds in E^3 (see *Summary*), so that in a sense these cases may be considered solved.

The use of polyhedral sets initially gave results in the topology of E^3 that constituted the only generalizations of simpler results known to hold in E^2. However, some of the techniques used in a 3-space when dealing with polyhedra are precisely

the ones that prevented progress in considering embedding problems in E^n, $n > 3$.

In 1958, B. Mazur gave conditions on the embedding of a topological $n-1$ sphere in E^3 that permitted a partial generalization of the Schoenflies theorem in the plane. His result may be properly regarded as a generalization of the Alexander result about polyhedral 2-spheres in E^3. In 1959, M. Brown gave an improved form of Mazur's result that reads: If a topological $n-1$ sphere T in E^n has a shell neighborhood, the bounded component of $E^n \setminus T$ (= complement of T in E^n) has a closure that is a closed n-cell.

In the present paper, T is supposed to be a topological image of a closed, combinational $n-1$ manifold in E^n. If T is locally unknotted in E^n (see *Definitions* below), then T has a shell neighborhood. In a 3-space, this yields nothing new, because, as was pointed out in the original definition,[3] local unknottedness of a 2-manifold in E^3 automatically implies the manifold to be locally tame, and the result of Bing and Moise applies.[1, 6]

In dimension 4, the result is believed to be new, and the hypothesis that T be a combinatorial manifold is unnecessary in view of Moise's work. The main result (Theorem 1), stated roughly above, is a partial generalization for E^n of the well-known and useful result in E^3 that locally tame sets are tame.

2. *Definitions and Notation.*—If C is a topological k-manifold in E^n, C is locally unknotted at x if and only if there is some $k+1$ cell D such that $C \cap D$ is the closure of a neighborhood of x in C that lies on a parameter k-cell spanning the boundary of D.[3] If C has this property at each point, call C locally unknotted (abbreviated $\mathfrak{LU}$).

If C is a topological k-manifold in E^n, C is locally peripherally unknotted at x if and only if for each positive ϵ there is a closed n-cell L of diameter less than ϵ whose interior contains x such that the common part of C with the boundary of L is a locally peripherally unknotted $k-1$ cell or sphere according as x lies on the boundary of C or not. If C has this property at each point, call C locally peripherally unknotted (abbreviated $\mathfrak{LPU}$).

A subset T of E^n has been called tamely embedded by Fox-Artin if and only if there is a homeomorphism of E^n on E^n carrying T onto a polyhedron.

Whitehead has called a topological k-sphere S^k tamely embedded in E^n if there is a homeomorphism of $S^k \times E^{n-k}$ into E^n such that S^k is the image of a standard $S^k \times [0]$.[7] (For $k \leq 3$, these definitions of *tamely embedded* agree when applied to S^k.)

A subset T of E^n is called locally tamely embedded in E^n at p provided there is a neighborhood N of p and a homeomorphism h_p of the closure of N ($= \bar{N}$) onto a polyhedron in E^n such that $h_p(\bar{N} \cap T)$ is a polyhedron (Bing and others). If T is locally tamely embedded at each point, it is locally tame (abbreviated $\mathfrak{LT}$).

If M^{n-1} is a closed, topological $n-1$ manifold in E^n and there is a homeomorphism of $M^{n-1} \times [-1, +1]$ into E^n such that $M^{n-1} \times [0]$ is the identity map, M^{n-1} is said to have a *shell neighborhood.*

This property and Whitehead's definition of tamely embedded, in contrast to the other definitions above, cannot be labeled a purely local or global property.

3. THEOREM. *Let M be a topological image of a closed, combinatorial $n-1$ manifold in E^n such that M is $\mathfrak{LU}$. Then M has a shell neighborhood.*

COROLLARY. *If A is the bounded component of E^n/M and M is an $n-1$ sphere, then the closure of A is a closed $n-1$ cell.* (Mazur,[5] Brown[2]).

Our methods are partly set-theoretic and partly combinatorial. The idea of the proof breaks into two parts. First, one notices that a generalization of the "well-canal-island" construction exploited in reference 4 (Proof of Theorem 1) is relatively straightforward for a combinatorial n − 1 manifold. Second, the use of polyhedral sets in generalization of the proof of Theorem 1 of reference 4 may be avoided by the use of the n-cells guaranteed by the definition of $\mathfrak{LU}$.

The definition of $\mathfrak{LU}$ and the existence of a triangulation on M are used to show that an arbitrary open subset of M may be homeomorphically pushed into either complementary domain in such a way as to move points an arbitrarily small amount.

Using this result and the fact that M is a topological n − 1 combinatorial manifold, one produces a partitioning of a neighborhood of M in E^n having a closure representable as a cell-complex that is isomorphic to a certain cell-complex on M. Finally, using the $\mathfrak{LU}$ property again, one is able to "fill in" the n-cells so as to produce the desired topological product.

4. *Summary.*—If $k = 1$ and $n = 2$, all k-manifolds in E^n are $\mathfrak{L}\mathfrak{Z}$, $\mathfrak{L}\mathcal{P}\mathfrak{U}$, and $\mathfrak{LU}$. If $k = 1$ and $n = 3$, the states $\mathfrak{LU}$ and $\mathfrak{L}\mathcal{P}\mathfrak{U}$ are independent. A 1-manifold is $\mathfrak{L}\mathfrak{Z}$ if and only if it is $\mathfrak{LU}$ and $\mathfrak{L}\mathcal{P}\mathfrak{U}$. For $k = 2$ and $n = 3$, being $\mathfrak{L}\mathfrak{Z}$ implies being $\mathfrak{LU}$, which in turn implies being $\mathfrak{L}\mathcal{P}\mathfrak{U}$. At an individual point, being $\mathfrak{L}\mathcal{P}\mathfrak{U}$ does not imply being $\mathfrak{LU}$, but if M is $\mathfrak{L}\mathcal{P}\mathfrak{U}$ (at every point), M is $\mathfrak{LU}$ and $\mathfrak{L}\mathfrak{Z}$.[4] For arbitrary n and $k = n - 1$, if M is $\mathfrak{LU}$ and is a combinatorial $n - 1$ manifold, it has a shell neighborhood; hence, if M is also an $n - 1$ sphere, the closure of its bounded component is an n-cell.

In view of the results for $k = 2$ and $n = 3$, it seems possible that if M is an $n - 1$ manifold that is $\mathfrak{L}\mathcal{P}\mathfrak{U}$ (instead of $\mathfrak{LU}$), it will turn out to be $\mathfrak{L}\mathfrak{Z}$.

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EXEMPLES DE VARIÉTÉS PROJECTIVES EN CARACTÉRISTIQUE p NON RELEVABLES EN CARACTÉRISTIQUE ZÉRO

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1. *Position du problème.*—La *théorie des schémas* de Grothendieck[1] permet de définir avec précision le terme de "relèvement":

Soit X_0 une variété projective non singulière, définie sur un corps k; soit A un anneau local noethérien complet de corps résiduel k. Un *relèvement de X_0 sur A* est un schéma X propre et plat sur A, tel que $X \otimes_A k$ soit isomorphe à X_0 (cf. Grothendieck[2]). Le cas qui nous intéresse ici est celui où k est de caractéristique p et A de caractéristique zéro. On pose la question suivante:

(1) *Peut-on toujours relever X_0 sur A?*

D'après Grothendieck,[2] la réponse est affirmative si X_0 est une courbe, ou, plus généralement, si certains groupes de cohomologie de X_0 sont nuls.

On peut également poser une question plus faible:

(2) *Pour une variété X_0 donnée, existe-t-il toujours un anneau A de caractéristique zéro sur lequel X_0 se relève?*

Nous allons montrer que *ces deux questions admettent une réponse negative, même si k est algébriquement clos.*

2. *Construction du contre-exemple.*—Soit n un entier $\geqslant 1$. Si R est un anneau local, nous noterons $\mathbf{GL}_n(R)$ le groupe des matrices inversibles d'ordre n à coefficients dans R, et $\mathbf{PGL}_n(R)$ le groupe quotient de $\mathbf{GL}_n(R)$ par le sous-groupe R^* des matrices scalaires.

Soit G un groupe fini, et soit r_0 un homomorphisme de G dans le groupe $\mathbf{PGL}_n(k)$. Le groupe G opère sur l'espace projectif $\mathbf{P}_{n-1}(k)$ au moyen de r_0. Nous ferons l'hypothèse suivante:

(*D*)—*Pour tout $\sigma \in G$, $\sigma \neq 1$, l'ensemble F_σ des points de $\mathbf{P}_{n-1}(k)$ invariants par σ est de codimension $\geqslant 4$.*

Un raisonnement classique, dû essentiellement à Godeaux,[3] montre qu'il existe une sous-variété non singulière Y_0 de $\mathbf{P}_{n-1}(k)$, stable par G, qui est une intersection complète, et qui ne rencontre aucun des F_σ, $\sigma \neq 1$; l'hypothèse (*D*) permet en outre de supposer que $\dim(Y_0) \geqslant 3$. Le groupe G opère librement (c'est-à-dire "sans points fixes") sur Y_0 et le quotient $X_0 = Y_0/G$ est une variété projective non singulière.

Lemme. *Si X_0 se relève sur A, l'homomorphisme $r_0 : G \to \mathbf{PGL}_n(k)$ se relève en un homomorphisme $r : G \to \mathbf{PGL}_n(A)$.*

Admettons provisoirement ce lemme. Pour obtenir un contre-exemple aux questions (1) et (2) du n^0 1, il suffit donc de construire un groupe G et un homomorphisme $r_0 : G \to \mathbf{PGL}_n(k)$ qui vérifie (*D*) et qui ne se relève à aucun anneau de caractéristique zéro. C'est là un problème de pure théorie des groupes, qui ne présente aucune difficulté. Supposons par exemple $p \geqslant 7$, et soit G un groupe de type $(p, \ldots, p)$. Prenons $n = 5$. Soit $N = (u_{ij})$ la matrice nilpotente d'ordre 5 définie par $u_{ij} = 1$ si $j = i + 1$, $u_{ij} = 0$ sinon; soit h un isomorphisme de G sur un sous-groupe du groupe additif de k, et soit $s(\sigma) = \exp(1 + h(\sigma)N)$. L'appli-

cation $\sigma \to s(\sigma)$ est un homomorphisme de G dans $\mathbf{GL}_5(k)$. Si $r_0(\sigma)$ désigne l'image de $s(\sigma)$ dans $\mathbf{PGL}_5(k)$, l'application $r_0 : G \to \mathbf{PGL}_5(k)$ est un homomorphisme. Si $\sigma \neq 1$, l'ensemble F_σ est réduit à un point, ce qui montre que (D) est satisfaite. Enfin, si r_0 se relevait en $r : G \to \mathbf{PGL}_5(A)$, on pourrait supposer A intègre (quitte à le remplacer par $A/\mathfrak{p}$, où $\mathfrak{p}$ est un idéal premier ne contenant pas le nombre p); on en déduirait l'existence d'un corps K de caractéristique zéro tel que $\mathbf{PGL}_5(K)$ contienne un sous-groupe isomorphe à G, ce que l'on voit facilement être impossible si l'ordre de G est $\geqslant p^5$.

3. *Démonstration du lemme.*—Soit X un schéma sur A relevant X_0. D'après un résultat de Grothendieck,[2] le groupe fondamental de X s'identifie à celui de X_0. Comme Y_0 est un revêtement étale ("non ramifié" dans l'ancienne terminologie) de X_0, galoisien et de groupe de Galois G, on en conclut qu'il existe un revêtement étale Y de X, jouissant des mêmes propriétés, et tel que $Y \otimes_A k = Y_0$. Soit $\mathcal{O}$ le faisceau d'anneaux de Y, et soit $\mathcal{O}_0$ celui de Y_0. D'après FAC n° 78, on a $H^0(Y_0, \mathcal{O}_0) = k$ et $H^1(Y_0, \mathcal{O}_0) = 0$. Il en résulte[4] que $H^0(Y, \mathcal{O}) = A$.

Soit $\mathcal{E}_0 = \mathcal{O}_0(1)$ le faisceau localement libre de rang 1 défini sur Y_0 par le plongement projectif $Y_0 \to \mathbf{P}_{n-1}(k)$. Comme $\dim(Y_0) \geqslant 3$, on a $H^2(Y_0, \mathcal{O}_0) = 0$ (FAC, *loc. cit.*) et un théorème de Grothendieck[2] montre qu'il existe sur Y un faisceau $\mathcal{E}$, localement libre de rang 1, et tel que $\mathcal{E} \otimes_A k = \mathcal{E}_0$; comme $H^1(Y_0, \mathcal{O}_0) = 0$, ce faisceau est unique, à un isomorphisme près.[2] D'autre part, d'après FAC, *loc. cit.*, on a $H^0(Y_0, \mathcal{E}_0) = k^n$ et $H^1(Y_0, \mathcal{E}_0) = 0$. Il en résulte[4] que $H^0(Y, \mathcal{E})$ est un A-module libre de rang n, et que $H^0(Y, \mathcal{E}) \otimes_A k$ s'identifie à $H^0(Y_0, \mathcal{E}_0) = k^n$. Si σ est un élément de G, l'unicité de $\mathcal{E}$ montre l'existence d'un isomorphisme $a(\sigma) : \mathcal{E} \to \mathcal{E}$ compatible avec $\sigma : Y \to Y$; cet isomorphisme définit un automorphisme de $H^0(Y, \mathcal{E}) = A^n$; de plus, $a(\sigma)$ est déterminé à la multiplication près par un automorphisme de $\mathcal{O}$, par un élément de A^*. On obtient donc ainsi un élément bien déterminé $r(\sigma)$ de $\mathbf{PGL}_n(A)$, et il est clair que l'application $r : G \to \mathbf{PGL}_n(A)$ est un homomorphisme qui relève r_0, cqfd.

4. *Compléments.*—(*a*) Si X_0 est la variété définie ci-dessus, on peut montrer qu'il existe un entier n tel que tout anneau sur lequel X_0 se relève soit annulé par p^n. En d'autres termes, *l'anneau local de la variété formelle des modules*[5] *de X_0 est annulé par p^n*. (*b*) On peut considérer X_0 comme un *cycle* dans un certain espace projectif. Le fait que X_0 ne se relève pas en tant que schéma montre qu'il ne se relève pas non plus comme cycle (au sens de la *réduction des cycles* de Shimura[6]).

[1] Grothendieck, A., et J. Dieudonné, "Eléments de Géométrie Algébrique," *Publ. Math. Inst. Htes. et Sci.* (Paris).

[2] Grothendieck, A., "Géométrie formelle et géométrie algébrique," *Sém. Bourbaki*, Mai 1959, no. 182.

[3] Voir par exemple J-P. Serre, "Sur la topologie des variétés algébriques en caractéristique p," *Symp. Top. Mexico*, 1956, pp. 24–53.

[4] Lorsque A est un anneau de valuation discrète, il suffit d'appliquer la *formule de Künneth* (cf. W. L. Chow et J. I. Igusa, "Cohomology theory of varieties over local rings," these PROCEEDINGS, **44**, 1958, pp. 1244–1248). Dans le cas général, il faut recourir au *théorème des fonctions holomorphes*, sous la forme cohomologique que lui a donnée Grothendieck (cf. le Chapitre III des "Eléments").

[5] Grothendieck, A., "Technique de descente et théorèmes d'existence en géométrie algébrique. II: Le théorème d'existence en théorie formelle des modules," *Sém. Bourbaki*, Février 1960, no. 195.

[6] Shimura, G., "Reduction of algebraic varieties with respect to a discrete valuation of the basic field," *Amer. J. of Math.*, **77**, 134–176 (1955).

*A FAVORABLE STRATEGY FOR TWENTY-ONE**

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1. *Introduction.*—It has long been an open question as to whether those of the standard gambling games which are not repeated independent trials admit strategies favorable† to the player. There have been numerous implications[2-4] that favorable strategies do not exist. In this note, we settle the issue by showing that there is a markedly favorable mathematical‡ strategy for one of the most widely played games, twenty-one, or blackjack.

2. *Previous Work.*—Our point of departure is the work of Baldwin, Cantey, Maisel, and McDermott,[5, 6] the only serious treatment of blackjack that has been given to date. The reader will find further references and a representative set of rules in their paper. Although there are minor variations in the game, we shall adopt those rules (including insurance[6]).

3. *Method and Results.*—Our calculations are similar to those outlined in Baldwin *et al.*,[5] but there are some very important changes. First, a high-speed computer was programmed to find the player's best possible strategy and the corresponding expectation. The electronic calculator enabled us to dispense with many of the approximations that were needed by Baldwin *et al.* to reduce the calculations to desk computer size. This led to noticeable improvements in results. In particular, the player's expectation for a complete deck was found to be a startling -0.21%. (Baldwin *et al.* give -0.62%). Our second change in approach was to program the computer to do the calculations for arbitrary sets of cards. This made it possible to take into account cards that become visible during play, a feature which is essential for the determination of any winning strategy.§

A standard deck of cards has approximately 3.4×10^7 subsets which are distinguishable under the rules of blackjack. It is thus impractical to compute the optimal strategy for each of these subsets. Instead, we have studied a number of carefully preselected subsets, and from the information gained, several favorable strategies are obtained. Some of our subsets and results are given in Table 1 below.

Let Q(I) be the number of cards of value I. The special subsets in Table 1 differ from a full deck only in that the number of cards of a single value has been altered.

In actual play, these special subsets occur infrequently, and some are even impossible. Even so, they yield a profusion of winning strategies. For example, one

TABLE 1

PLAYER'S EXPECTATION WITH SELECTED SUBSETS

Description of the subset	Player's expectation	Description of the subset	Player's expectation
complete deck	−.0021	Q(7) = 0	.0125
Q(1) = 0	−.0272	Q(8) = 0	.0005
Q(2) = 0	.0142	Q(9) = 0	−.0091
Q(3) = 0	.0189	Q(10) = 12	−.0215
Q(4) = 0	.0236	Q(10) = 20**	.0189
Q(5) = 0	.0329	Q(10) = 24**	.0394
Q(6) = 0	.0187		

of these winning strategies may be obtained by considering the subset Q(5) = 0. Suppose that just before a particular deal the player sees that all fives have been used (so that the unseen cards are a subset of that subset which we describe by Q(5) = 0) and that the unused portion of the deck is ample for that deal. If the player does not take into account the cards other than fives that he has seen on previous deals, then as far as he is concerned, his probabilities for success are the same as for the subset Q(5) = 0. Using Table 1, it follows that the player who adopts the strategy for Q(5) = 0 (see Table 2) when there are no fives remaining has an expectation of 0.0329 at those times.

A winning strategy may now be defined as follows. If Q(5) $\neq$ 0, the player bets the minimum allowed amount, m, merely to remain in the game and follows the complete deck strategy given by Baldwin *et al.* When Q(5) = 0 (and the remainder of the deck will suffice for the next deal), the situation has turned in favor of the player. He now bets a large amount, M, and uses the computed strategy for Q(5) = 0, which is given in Table 2.††

TABLE 2

THE STRATEGY WHEN Q(5) = 0

Pair Splitting
Dealer shows:

Pair	2	3	4	6	7	8	9	10	A
A	X	X	X	X	X	X	X	X	X
10				X					
9	X	X	X	X	X	X	X		
8	X	X	X	X	X	X	X	X	X
7	X	X	X	X	X	X	X		
6	X	X	X	X					
4			X						
3	X	X	X	X	X	X			
2	X	X	X	X	X	X			

Doubling Down
Dealer shows:

Total	2	3	4	6	7	8	9	10	A
20				S					
19		S	S	S					
18	S	S	S	S					
17	S	S	S	S	S				
15			S	S					
14		S	S	S					
13		S	S	S					
11	H	H	H	H	H	H	H	H	H
10	H	H	H	H	H	H	H	H	H
9	H	H	H	H	H				
8			H	H					

Minimum Standing Numbers
Dealer shows:

Total	2	3	4	6	7	8	9	10	A
19							S	S	
18	S	S	S	S	S	S			S
17									H
16				H	H				
15							H	H	
12	H	H	H	H					

Legend:

X: split the pair
S: soft total only
H: hard total only

A disadvantage of this strategy is that the event Q(5) = 0 occurs only in about 3.5 per cent to 10 per cent of the deals (depending on the number of players). A similar remark applies to the other Q(I) = 0 type strategies. However, careful scrutiny has disclosed another strategy which partially overcomes this disadvantage and gives the player a greater expectation as well. This strategy depends on the somewhat surprising fact that all the crucial quantities are almost linearly dependent on the proportion of tens in the deck and nearly independent of the absolute number of tens. The details are too extensive to be given here and will appear elsewhere. The main characteristics are that the player has an advantage

almost half the time; his expectation exceeds 0.04 about a tenth of the time, and occasionally (probability 1/5,000–1/10,000) exceeds 0.86.

Since this strategy offers a "spectrum" of favorable expectations, it seems reasonable to let the size of the bets increase with the expectation. The advantages of this procedure have not yet been studied in detail. Nor have we considered in detail the question of letting the player's bet vary with the size of his fortune.

4. *Remarks.*—With only minor modifications, our program can be used by a high-speed computer to play blackjack directly. The computer would play a near-perfect game. If the bets were of constant size, the player would have a decided advantage. If the bet size were varied, the player's advantage would be overwhelming.

The rules variations in Nevada casinos have been tabulated and analyzed. The expectations never vary more than 0.005 from our figures. Consequently our strategies are advantageous regardless of these variations in rules.

The "home" game of blackjack differs from the casino game principally in that the dealer's strategy is fixed in the latter and arbitrary in the former. Using our methods, it is an easy matter to find the optimal player strategy against each possible fixed dealer strategy. The methods of game theory then apply to the case of mixed dealer strategies and yield a complete solution for the home game.

The author is indebted to R. Baldwin, W. Cantey, H. Maisel, and J. McDermott for making available their detailed calculations and to the M.I.T. Computation Center for making the IBM 704 available.

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† A strategy is *favorable* if, for some uniform bound on the player's bets, his fortune converges with probability 1 to plus infinity.

‡ By saying "mathematical strategy," we mean to exclude such time-honored approaches to winning strategies as physical strategies (defective roulette wheel, defective dice) or the large class of strategems (sleight of hand with the cards, collusion with the dealers, etc.).

§ Further detailed results, together with the lengthy computer routine, the methods used to insure that it is correct, and the discussion of the errors introduced by certain simplifying assumptions, will appear elsewhere.

** Insurance contributes 0.0032 to this value when $Q(10) = 20$ and 0.0073 when $Q(10) = 24$.

†† At the casinos, M/m generally is from 100 to 500. With these values, the overall "expectation" E (i.e., the expected value of the amount won, in units of M, divided by the number of times that M was bet) is greater than 0.03. In fact, $M/m \geqq 15$ insures $E \geqq 0.025$ and if $M/m \geqq 3$, $E > 0$.

[1] Feller, W., *An Introduction to Probability Theory and Its Applications* (New York: John Wiley and Sons, Inc., 1957).

[2] Huff, Darrell, "*The Mathematics of Sex, Gambling, and Insurance*," *Harper's*, Sept. 1959.

[3] Fox, Philip G. (as told to Stanley Fox), "A Primer for Chumps," *Sat. Eve. Post*, Nov. 21, 1959, pp. 31ff.

[4] Von Mises, Richard, *Probability, Statistics and Truth*, (London: Allen and Unwin, 1957).

[5] Baldwin, R., W. Cantey, H. Maisel, and J. McDermott, "The Optimum Strategy in Blackjack," *J. Am. Stat. Assn.*, **51**, 429–439 (1956).

[6] Baldwin, R., W. Cantey, H. Maisel, and J. McDermott, *Playing Blackjack to Win: A New Strategy for the Game of 21* (New York: M. Barrows, 1957).

STRENGTHENING OF SPERNER'S LEMMA APPLIED TO HOMOLOGY THEORY

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The theorem below, a strengthened form of Sperner's Lemma, makes possible a short proof, making no use of the so-called prism cells, of the property that a j-cycle composed of cells of a complex K and bounding a $(j+1)$-chain C^{j+1} on $|K|$ bounds a $(j+1)$-chain D^{j+1} of cells of K. The proof that the homology group of the j-cycles on $|K|$ is isomorphic to that determined by the cells of K is easily completed by use of prism cells in an argument in which j-cycles and $(j+1)$-chains appear but no $(j-1)$-chains. The proof is shorter and simpler than that originally given by J. W. Alexander. Full details of the application to homology theory will appear in Cairns' forthcoming *Introductory Topology* (Ronald Press). The strengthening of Sperner's Lemma was obtained by Brown when reading the manuscript of the book.

Theorem. *Given an oriented n-simplex s^n, suppose that $K(s^n)$, the complex of $\bar{s}^n$, is subdivided into a linear simplicial complex K^n whose n-simplexes are oriented like s^n. Let f be a mapping of the 0-cells of K^n onto the vertices of s^n under which the image of each 0-cell of K^n lies on the closure of the simplex of $K(s^n)$ on which it lies. Then the number of n-simplexes of K^n whose vertices are mapped onto all $n+1$ vertices of s^n with preservation of orientation exceeds by one the number whose vertices are mapped onto all $n+1$ vertices with reversal of orientation.*

Proof. Using mathematical induction, take $n=1$, so that $s^n = s^1$ is an oriented 1-cell and let $\partial s^1 = s_1^0 - s_0^0$. Let $\{t_i^1\}$ be the 1-cells of K^1, oriented like s^1. Then $f(\sum_i \partial t_i^1) = f(s_1^0 - s_0^0) = s_1^0 - s_0^0$. Now suppose that x of the t_i^1 have end points mapped onto s_1^0 and s_0^0 so that orientation of the 1-cell is preserved, that y are mapped with orientation reversed, and that z have both end points mapped into s_1^0 or both mapped into s_0^0. Then,

$$f(\sum_i \partial t_i^1) = x(s_1^0 - s_0^0) + y(s_0^0 - s_1^0) + z(0) = (x-y)(s_1^0 - s_0^0).$$

Hence $s_1^0 - s_0^0 = (x-y)(s_1^0 - s_0^0)$, from which we infer that $x - y = 1$, completing the proof for $n = 1$.

Now assume that the lemma has been proved for $n = 1, 2, \ldots, r$. Let $s_0^r, s_1^r, \ldots, s_{r+1}^r$ be the boundary r-simplexes of s^{r+1}, with the orientations induced by that of s^{r+1}. Let $\{t_i^{r+1}\}$ be the $(r+1)$-simplexes of K^{r+1}, oriented like s^{r+1}, oriented like s^{r+1}, and let $\{u_j^r\}$ be the r-simplexes of K^{r+1} which are on $s_0^r, \ldots, s_{r+1}^r$, each oriented like the one of the latter on which it lies.

With the mapping by f of the vertices of any oriented simplex of K^{r+1} we associate in an obvious way a mapping of the oriented simplex itself.

Then $f(\partial\sum_i t_i^{r+1}) = f(\sum_j u_j^r) = \sum_j f(u_j^r)$, which, by the theorem for $n = r$, equals $(s_0^r + s_1^r + \ldots + s_{r+1}^r)$ + degenerate r-simplexes.

On the other hand, $f(\partial\sum t_i^{r+1}) = \sum f(\partial t_i^{r+1})$. Now if the number of distinct

O-cells among the images under f of the vertices of t_i^{r+1} is exactly $r + 1$, it is easily verified that $f(\partial t_i^{r+1})$ contains exactly two non-degenerate r-simplexes of opposite orientations, so that $f(\partial t_i^{r+1}) = O +$ degenerate r-simplexes. If the number is less than $r + 1$, there are no nondegenerate r-simplexes in $f(\partial t_i^{r+1})$. Hence, the only cases in which $f(\partial t_i^{r+1}) \neq O$, when degenerate r-simplexes are dropped, are those in which the $r + 2$ vertices of t_i^{r+1} are mapped onto the $r + 2$ vertices of s^{r+1}. Suppose that this happens for x of the t_i^{r+1} with orientation preserved and for y with orientation reversed. Then $\sum_i f(\partial t_i^{r+1}) = (x - y)(s_0^r + s_1^r + s_{r+1}^r)$, and, as in the proof for the case $n = 1$, we infer that $x - y = 1$. This completes the proof.

RIBOSOME-BOUND β-GALACTOSIDASE

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Considerable evidence has been accumulated to show that ribonucleoprotein particles (ribosomes) provide sites for protein synthesis in animal cells.[1] Studies of the incorporation of radioactive tracers by *Escherichia coli* also indicated that the ribosomes of bacteria are active in protein synthesis (McQuillen, Roberts, and Britten[2]). These authors showed that in growing bacteria, a quantity of material roughly equal to the protein synthesized in three seconds was transiently associated with the ribosomes before being released to the soluble protein fraction of the cell. Since the rate of protein synthesis in *E. coli* is about 0.02 per cent per second, 0.06 per cent of any *particular* protein might be expected to be found transiently associated with the ribosomes. A series of experiments was started to determine whether β-galactosidase showed the same transient association with ribosomes as was indicated for proteins in general. The first experiments showed that a small fraction of the enzyme was bound to the ribosomes, and it is the purpose of the present paper to describe some of the properties of this ribosome-associated enzyme. Furthermore, the results suggest a general procedure for isolating specific ribosomes.

Materials and Methods.—E. coli strains: Three strains of *E. coli* differing in their β-galactosidase-synthesizing properties were used, namely ML 30 (inducible), ML 308 (constitutive), and W2214 (absolute negative).

Growth conditions: Cells were grown at 37°C in a vigorously aerated synthetic medium (C) of the following composition: 2 *g* NH_4Cl, 6 *g* Na_2HPO_4, 3 *g* KH_2PO_4, 3 *g* NaCl, 0.01 *g* Mg as $MgCl_2$, 0.026 *g* S as Na_2SO_4, 900 ml H_2O, and 10 ml. of 10 per cent maltose.

Enzyme induction and assay: Thiomethyl-β-D-galactoside (TMG) or thioisopropyl-β-D-galactoside (TIPG) at 5×10^{-4} *M* were used as inducers of β-galactosidase synthesis. Assays of β-galactosidase on ribosomal preparations were performed with the following mixture buffered at pH 7.4; 0.0027 *M* ortho-nitrophenyl-β-D-galactoside (ONPG), 0.05 *M* NaCl, 0.01 *M* trishydroxymethylaminomethane

(Tris), 0.004 *M* succinic acid, and 0.01 *M* magnesium acetate. The hydrolysis of the ONPG was followed in a Beckman spectrophotometer at 420 mμ. The sodium chloride, tris-succinate mixture was used in place of the more commonly employed phosphate buffer because of the known[3] instability of ribosomes in the presence of phosphate. The rate of ONPG hydrolysis is the same in the two buffer systems.

Preparation of wall-free cell juice: Exponentially growing cultures of *E. coli* were harvested and washed once in a Tris buffer adjusted to pH 7.6 containing 0.01 *M* Tris, 0.004 *M* succinic acid, and 0.01 *M* magnesium acetate (TSM). Following the wash, the cells were resuspended in 10 ml of the same buffer. The cells

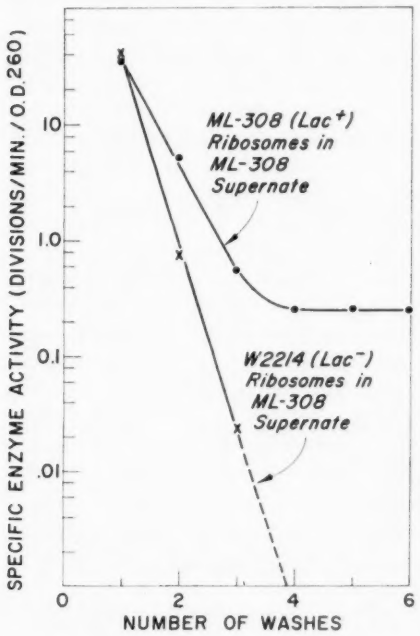

FIG. 1.—The effect of successive washings by centrifugation on the specific enzyme activity of ribosomes from genetic positives (●) and ribosomes from genetic negatives (X), both initially suspended in an extract containing large amounts of active β-galactosidase.

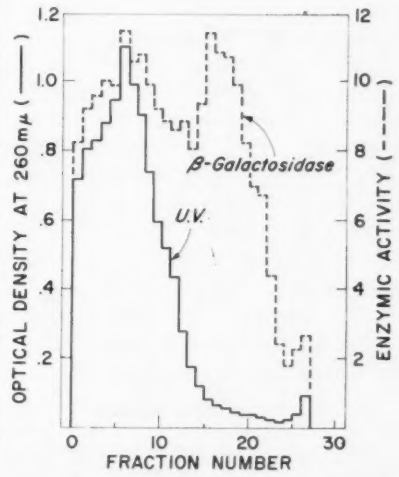

FIG. 2.—Sedimentation pattern of Lac+ ribosomes washed three times prior to being layered on the sucrose gradient (3 to 20 per cent). The run was made at 37K for 80 min.

of this suspension were ruptured by extrusion through a small orifice under pressure (approximately 15,000 lb/sq. inch) in a modified French pressure cell. The extruded material was centrifuged for five minutes at 40,000 rpm in the angle head rotor of the Spinco Model L centrifuge to remove whole cells, cell walls, and other large fragments.

Antisera: β-galactosidase antiserum was prepared by injecting rabbits with 10 mg of purified (90 per cent) *E. coli* β-galactosidase. Chicken anti-rabbit serum was kindly furnished us by Dr. Alan Boyden, Rutgers University, New Brunswick, N. J.

Results.—Enzymatic activity of ribosomes: Centrifugation of a wall-free cell

juice for 45 minutes at 40,000 rpm in the angle head rotor of the Spinco Model L centrifuge gives a pellet (40K 45P) which contains more than 90 per cent of the 70S and 85S ribosomes of the cell. Such a centrifugation leads to a useful separation of the major ribosome components from the bulk of the soluble proteins and smaller particles and yields material suitable for further purification.

One technique for purifying large quantities of 85S and 70S ribosomes involves repeated washing in TSM. Figure 1 compares the amounts of enzymic activity found in the pellet fractions (40K 45P) in successive washings of ribosomes derived from ML 308, the constitutive mutant (circles). It will be noted that by the fourth washing a constant specific enzymic activity (enzyme units per O.D. at 260 mμ) is achieved.

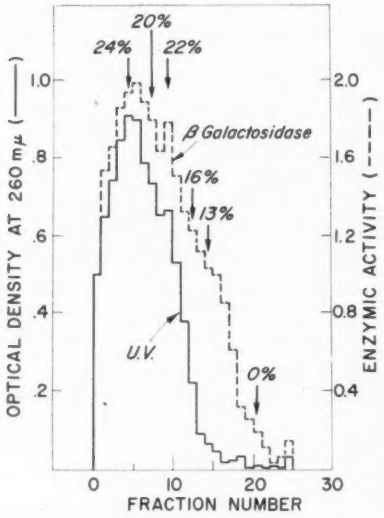

FIG. 3.—Sedimentation pattern of the ribosomes in the first 13 fractions of Figure 2. The numbers denote per cent increases in enzyme activity when the corresponding fraction was incubated with anti-β-galactosidase serum.

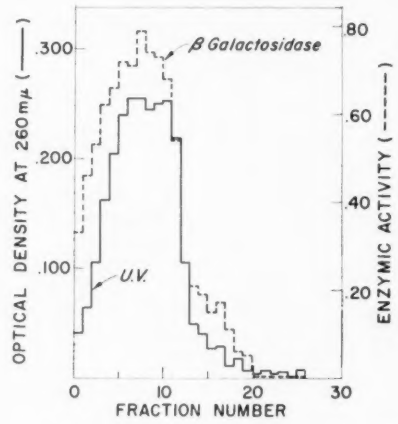

FIG. 4.—Sedimentation pattern of the ribosomes in the first 13 fractions of Figure 3.

Such data do not necessarily establish a specific association between a fraction of the enzyme molecules and the ribosomes. For example, some enzyme molecules could be nonspecifically but firmly adsorbed to the surface of the ribosomes. Alternatively, the enzymic activity of the pellet may not be associated with the ribosomes but may reside in aggregates possessing an average sedimentation constant roughly equivalent to the larger ribosomes.

The first of these possibilities was excluded by the following experiment. An extract rich in enzyme but free of ribosomes was prepared from the constitutive strain, ML 308 (Lac^+), by centrifuging the cell juice for five hours at 40,000 rpm. Purified ribosomes prepared from the absolute lactose-negative strain W2214 (Lac^-) were suspended in this extract. The resulting mixture was subjected to the

successive centrifugations in TSM. Figure 1 shows that the enzyme associated with the genetically negative ribosomal pellet behaves quite differently from that observed with genetically competent material. Initially, the specific enzymic activities of the two ribosomal pellets are about equal. In the lower curve, there is no suggestion of an approach to a constant specific activity on repeated washings. By the fourth step, the Lac^- ribosomes reach a specific activity (not indicated on the graph) which is less than 0.001 units of enzyme per optical density unit. Such enzymic activities are too low for accurate measurement and correspond to levels considerably less than 1/100 of the constant specific activities attained with the Lac^+ ribosomes.

Centrifugation of a layer of ribosome suspension through a sucrose gradient in the swinging bucket rotor of the Spinco centrifuge[4] provides a much better indication that the enzyme is actually associated with the ribosomes.

Figure 2 shows the distribution of the enzymic activity and optical density of a ribosome preparation (subjected first to two washes as described above) after being layered on the sucrose gradient and centrifuged for 45 minutes at 37,000 rpm. The enzyme is distributed roughly equally between the ribosomal and supernatant fractions. Figures 3 and 4 illustrate how successive sedimentations through sucrose gradients lead to the elimination of the soluble enzyme component from the ribosome fraction. The first 13 fractions of the run described in Figure 2 were pooled and the ribosomes collected by centrifugation (40K 45P). They were then resuspended and layered on a new sucrose gradient and centrifuged. The resulting profile of O.D. at 260 mμ and of enzyme activity is given in Figure 3. Comparatively little enzyme is found unassociated with the ribosome peak although there is clearly a contamination of free enzyme. A repetition of this procedure on the first 13 fractions of Figure 3 is shown in Figure 4. Here, there is an excellent correlation of enzyme activity with ribosome peaks in the density gradient.

Nature of the ribosome-associated enzyme: The sedimentation patterns described above encouraged the belief that some enzyme molecules are in physical association with the larger ribosome particles. It was of interest to look for some feature other than the sedimentation characteristics which would serve to distinguish these molecules from those which are in the soluble fraction. A series of experiments were, therefore, performed examining the effect of a variety of agents on the residual ribosomal enzyme activity. Methods of disrupting ribosomes (RNAase, versene, or citrate) which have been shown to be capable of releasing latent RNAase activity of the ribosomes of *E. coli*[5, 6] did not cause any increase in the β-galactosidase activity of highly purified ribosomal preparations. Several attempts to activate such preparations by the addition of the galactoside inducers, TMG and TIPG, were also unsuccessful.

The effect of adding specific antiserum was examined with the hope that it might serve as a specific means for removing the associated enzyme from the ribosomes. Table 1 shows that exposure of purified ribosomes to anti-β-galactosidase results in a striking increase in enzymic activity. In a series of similar experiments using comparably purified ribosomes, increases of between four- and sixfold were often observed. This activation is unique for the specific anti-β-galactosidase serum; normal rabbit serum and nonhomologous antisera (e.g. anti-alkaline phosphatase) have no effect. Further, the increase in activity is confined to ribosome-bound

TABLE 1

EFFECT OF RABBIT SERUM UPON RIBOSOME ASSOCIATED AND SOLUBLE β-GALACTOSIDASE

Fraction	Rabbit serum added	Enzyme activity
Ribosomes (uninduced ML 30)	0	0.04
Ribosomes (" ")	Anti-β-galactosidase	0.15
Ribosomes (" ")	Normal	0.05
Soluble (" ")	0	0.092
Soluble (" ")	Anti-β-galactosidase	0.090

Ribosome fractions were purified by successive centrifugations to constant specific activity and enzyme was assayed with ONPG according to the procedures described under *Methods*. Soluble enzyme was obtained by removal of ribosomes by means of a sucrose gradient swinging bucket centrifugation. Assays were continued until linear rates were well established.

enzyme and is not observed with the soluble enzyme as shown in Figure 3. Aliquots taken from fractions corresponding to different portions of this O.D. profile were tested for their ability to be activated by the specific β-galactosidase antiserum. The increases over controls are recorded in Figure 3 over the appropriate parts of the profile.‡ Fractions corresponding to the ribosome region exhibit the antiserum-activating effect. As one proceeds to fractions closer to the free soluble region, however, the degree of activation falls and finally becomes zero. Thus, the soluble enzyme and the enzyme associated with the ribosomes differ not only in their sedimentation rates but also in their response to antibody.

Two other possible explanations for such activation are (1) that the addition of ribosomes activates in some manner β-galactosidase present in the antiserum which is not detectable in their absence or (2) that the presence of ribosomes nonspecifically inhibits the activity of β-galactosidase and this inhibition can be reversed with specific antiserum. To test the validity of the first suggestion, purified ribosomes prepared from the Lac$^-$ strain (W2214) were incubated with the anti-β-galactosidase serum. These mixtures were assayed for enzymic activity and none was found. It is concluded from such experiments that the observed increase in activity is not due to a latent enzyme in the antiserum. To examine the second possibility, the following experiment was performed. A soluble enzyme fraction was prepared from fully induced cells by exhaustive centrifugation of the cell-free extract to remove the ribosomal components. Purified ribosomes from a Lac$^-$ strain of *E. coli* (W2214) were introduced into this soluble enzyme extract. The ribosomes were then purified in the usual fashion by successive centrifugations. The purification was stopped when the specific enzymic activity corresponded to that at which pronounced activation by antiserum is observed with Lac$^+$ ribosomes (see Table 1). The resulting mixture was then exposed to antiserum and the enzyme activity measured. No activation of the enzyme was ever observed. In a typical experiment, 0.092 div./min were observed in the absence of antiserum and 0.090 div./min in its presence. Since the Lac$^-$ ribosomes did not activate or depress the soluble enzyme activity, one is inclined to believe that the phenomenon is unique for ribosome-associated enzyme.

Mechanism of antibody action: Antibody was added to the ribosome preparations with the hope that the antibody might dislodge the enzyme molecule from the ribosome and thereby expose the active regions. A second possibility was that the antibody might help to shape the associated polypeptide without removing it from the ribosome. The sedimentation characteristics of the enzyme-antibody complex were therefore examined.

If the antiserum removes the enzyme from the ribosomes, one should no longer observe a peak of enzymic activity in the ribosome region. This possibility was tested under various conditions differing widely with respect to the amount of soluble β-galactosidase present. In one case, a soluble protein fraction from the constitutive mutant, containing a small quantity of ribosomes, was incubated with antiserum at room temperature for several hours. The amount of antiserum added was greatly in excess of that required to precipitate all the enzyme present. The mixture was then layered on a sucrose gradient and centrifuged at 37K for 90 minutes. A companion tube was run with enough purified ribosomes to show the position of the ribosome peak by its O.D. at 260 mμ. Figure 5 shows the distribution of enzymic activity observed (dashed line) and of the O.D. at 280 mμ (light line) indicative of the protein distribution. Superimposed is the distribution of O.D. at 260 (heavy line) obtained in the companion tube. More than 99 per cent of the enzyme activity was precipitated as a pellet, and virtually none was found in the region corresponding to the soluble protein. There is, however, still a peak of enzyme activity in the region corresponding to the ribosomes.

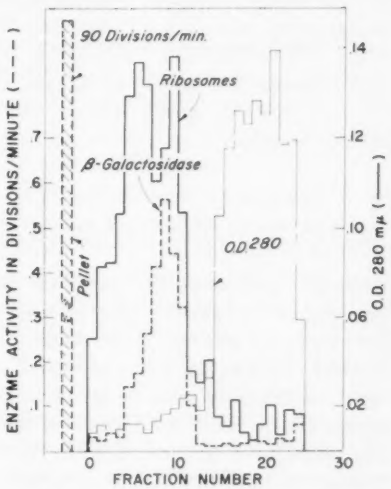

FIG. 5.—The effect of anti-β-galactosidase serum on the distribution of enzyme activity in a sucrose gradient sedimentation pattern of a small amount of ribosomes in the presence of excess enzyme. A companion tube was run to provide the position of the ribosomes as given by heavy line histogram.

The response of the ribosome-enzyme complex to antiserum was also examined using ribosome preparations at various stages of purification. In all cases, the same pattern was observed. Any soluble enzyme present was removed as a pellet, but there remained a residual peak of enzyme activity in the sedimentation pattern regions where the 70S and 85S ribosomes are located.

It is evident that antibody easily forms large precipitable aggregates with soluble enzyme but not with the ribosomal enzyme. Further, the activation effect of anti-serum does not seem to be produced by a removal of the enzyme molecules from their association with ribosomes. Were this the case, the enzyme either would precipitate rapidly, as does the soluble enzyme-antibody complex, or would show a decreased sedimentation constant if it were detached but not able to form an aggregate.

The association of the antibody with the ribosomes was demonstrated by the use of another antibody against the rabbit γ-globulin. A purified ribosome preparation was incubated for one hour in the presence of excess rabbit anti-β-galactosidase serum and then centrifuged for one minute at 25K. As expected, no detectable enzyme was removed from the supernatant, enzyme activities of the supernatants being 4.2 before and 4.7 per 0.1 ml after the centrifugation. In a control incubation and centrifugation with soluble enzyme, virtually all enzymic activity was removed

from the supernatant. Subsequently, excess chicken anti-rabbit serum was added to the mixture of ribosomes and anti-β-galactosidase rabbit serum. After an hour's incubation, this mixture was subjected to a centrifugation (25K for 1 minute). Examination of the supernatant revealed that 94 per cent of the ribosome-bound enzyme had been precipitated. The enzyme removed from the supernatant was found in the pellet fraction. Examination of the ribosome content of the supernatant revealed that less than one per cent had been removed. Hence, less than one per cent of the ribosomes possess the β-galactosidase enzyme.

The amount of ribosome-associated enzyme in different states of induction: It was of interest to examine the effect of induction on the amount of β-galactosidase found associated with the ribosome fraction. Table 2 summarizes the results of a

TABLE 2

β-GALACTOSIDASE ACTIVITY ASSOCIATED WITH RIBOSOMAL PARTICLES OF *E. coli*

Strain	Divisions per minute per O.D. unit at 260 mμ		
	ML 30 (basal)	ML 30 (induced)	ML 308 (constitutive)
	0.017	0.34	0.21
	0.018	0.60	0.17
	0.020	0.46	0.25
	0.016	0.46	0.23
Average	0.018	0.47	0.22

Ribosome fractions were purified by successive centrifugations to constant specific activity and enzyme was assayed with ONPG according to the procedures described under *Methods*.

series of experiments examining the enzymic activity of ribosomes prepared from cultures of noninduced, fully induced, and constitutive cells. The ribosomes were prepared and purified to constant specific enzymic activity by the methods described. It is evident that increased capacity to synthesize enzyme is accompanied by a considerable increase in the amount of ribosome-bound enzyme. A 25-fold increase in the enzymic activity of the ribosome fraction is observed in going from the noninduced to the fully induced state. During this same period, the enzyme content per cell increases by a factor of about 200. It is of interest to note that although the constitutive strain synthesizes about five times as much enzyme per cell as a fully induced cell, this difference is not reflected in an increased amount of ribosome-bound enzyme. On the contrary, this difference was consistently less by a factor of two in the constitutive mutant as compared with the inducible variety.

Discussion.—The experiments described provide strong evidence that the ribosomes carry a small fraction of the β-galactosidase of the cell, possessing three characteristics which distinguish it from the free enzyme molecules: (*a*) it sediments much more rapidly, possessing a sedimentation coefficient of about 70S; (*b*) the ribosome-bound activity is increased by exposure to specific antibody; (*c*) it is not precipitated by an antiserum which removes all of the soluble enzyme present in the reaction mixture. From the results described above, it is not possible to decide whether the enzyme is transiently or permanently associated with the ribosome in the living cell. Experiments to be reported later suggest that both classes are present.

It is of some interest to consider briefly the numerical relations of the partitioning of enzyme molecules between the ribosomal and soluble fractions. For such calculations, we assume that the molecular weight of the RNA in the 70S ribosomes is

1.8×10^6 and that 1 mg of ribosomal RNA/ml will have an optical density of 24. Thus, a solution of O.D. equal to 1 will contain 1.4×10^{13} 70S particles. The turnover number of purified β-galactosidase, measured with orthonitrophenyl-β-D-galactoside, is 4,000 moles/sec per mole of enzyme.[7] Under the conditions of assay, 1 $m\mu M$ of orthonitrophenol yields an O.D. of 0.000143 at 420 mμ. Using these numbers, one can calculate the number of ribosomes per enzyme molecule from the specific activities of the purified ribosomal preparations. This computation leads to a value of 5×10^3 ribosomes per enzyme molecule for the inducible strain in the noninduced state. Cells growing in C-medium are estimated to contain approximately $5–10 \times 10^3$ large ribosomes per cell. It is concluded that there is approximately one enzyme molecule per cell bound to the ribosomes in the noninduced strain. This increases by an order of magnitude upon full induction.

The kinetics of this increase are of great interest and are being investigated. The available data suggest that the increase is a fast process completed in about three minutes. The relative rate of enzyme synthesis in constitutive mutants is usually between 5 and 10 times that seen in fully induced inducible strains. It is evident (Table 2) that this higher rate cannot be ascribed to a higher content of ribosomes which can specifically retain β-galactosidase activity.

It must be emphasized that it is difficult to make unambiguous estimations of the absolute activities of the ribosome-bound enzyme molecules. It seems likely, however, that the orders of magnitude computed from these assays can be taken seriously. It is of some interest that the numbers obtained are what might have been expected from a reasonably simple interpretation of ribosome function. If the ribosomes carrying a given protein in association are the only ones concerned with its synthesis, one would conclude that one to ten ribosomes may be active at any given moment in the synthesis of a particular protein. Thus, the several thousand ribosome of an *E. coli* cell could synthesize roughly a thousand different enzymes which should be adequate to carry out the various cell functions. The data described, however, do not eliminate the possibility that a given ribosome may be concerned with the synthesis of a variety of proteins at different times of its existence.

The experiments reported here raise the question of whether other enzymes can be found associated with the ribosomes and whether these also possess similar distinguishing features. Preliminary experiments along these lines in *E. coli* indicate that the same situation obtains with a variety of enzymes. One in particular, alkaline phosphatase, for which an antiserum was available, also exhibited the antibody-activating effect. Halvorson[8] and his co-workers have uncovered a ribosome-bound fraction of β-glucosidase in yeast. It is again of interest to note that these investigators compute from their data that there is approximately one enzyme molecule bound to the ribosome fraction per cell.

It is evident that the use of specific antiserum may greatly aid the preparation of a ribosome suspension containing a particular protein in bound form and free of its soluble counterpart. Furthermore, the possibility exists of developing a general procedure for isolating ribosomes associated with specific proteins. This is suggested by the experiments in which the ribosome-bound enzyme was precipitated by first complexing with rabbit anti-β-galactosidase and then exposing the complex to an antibody directed against the rabbit γ-globulin.

Summary.—Experiments are described which provide evidence that a certain fraction of the β-galactosidase molecules of the cell are carried on the ribosomes. This fraction corresponds (in order of magnitude) to one molecule per cell in non-induced, inducible cells and rises to between 10 and 20 per cell for the fully induced and constitutive states. In addition to possessing an apparent higher sedimentation coefficient, the ribosome-bound enzyme molecules are distinguishable from their soluble counterparts in their response to specific anti-β-galactosidase serum. Antiserum precipitates the soluble enzyme without affecting the observed activity. Exposure of the ribosome-bound enzyme to antiserum results in a three- to sixfold rise in activity which is not accompanied by the formation of a precipitable aggregate. It was found that the complex can, however, be precipitated by the addition of an antiserum (chick anti-rabbit) directed against the antibody. The latter reaction suggests a means for the isolation of specific ribosomes.

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‡ Aged preparations lose their ability to respond to the specific antibody. One preparation which showed a fourfold increase in the presence of antibody showed no increase after two weeks storage at −20°C.

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*ON THE BOUND STATES OF A GIVEN POTENTIAL**

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It is a fundamental property of any spherically symmetrical potential V(r) for which $\int_0^\infty dr\ r|V(r)|$ exists that there are only a finite number of bound states. This has been expressed by V. Bargmann[1] in the form of the inequality[2]

$$(2l + 1)n_l < \int_0^\infty dr\ r|V(r)|$$

where n_l is the number of bound states for given l (and magnetic quantum number m_l). The method of derivation of the latter result is sufficiently specialized, however, that it is not easily applied to nonspherically-symmetrical potentials, for

example, or to the tensor forces that appear in the two-nucleon problem. Accordingly, we propose to give another derivation of this inequality, together with some of its extensions.

Any discussion of this subject has as its foundation the elementary fact that a decrease of the potential in some region must lower the energies of the bound states and therefore cannot lessen their number. Thus, we conclude that

$$n_l(V) \leq n_l(-|V|)$$

since the substitution of $-|V(r)|$ for $V(r)$ can either leave the potential unchanged or decrease it. Next, let us replace $-|V(r)|$ by $-\lambda|V(r)|$ with $0 < \lambda \leq 1$. An increase of λ lowers the energies of the bound states and cannot lessen their number. As λ increases from 0, we reach a critical value, λ_1, at which a bound state first appears at $E = -0$. With further growth of λ, the energy of this state decreases until we reach a second critical value, λ_2, at which a second bound state appears, and so on. When λ has attained the value unity and

$$\lambda_n \leq 1 < \lambda_{n+1},$$

there are n bound states.

The eigenvalue problem for λ, associated with $E = 0$ and orbital angular momentum l, is given by

$$\left(-\frac{d^2}{dr^2} + \frac{l(l+1)}{r^2}\right) u_l(r) = \lambda|V(r)|u_l(r)$$

or, on incorporating the boundary conditions,

$$u_l(r) = \lambda \int_0^\infty dr'\, g_l(r, r')|V(r')|u_l(r').$$

The Green's function, $g_l(r, r')$, obeys

$$\left(-\frac{d^2}{dr^2} + \frac{l(l+1)}{r^2}\right) g_l(r, r') = \delta(r - r')$$

and is explicitly given by

$$g_l(r, r') = \frac{1}{2l+1} r_<^{l+1} r_>^{-l}.$$

The integral equation can also be written with a real, symmetrical kernel,

$$\lambda^{-1}\phi_l(r) = \int_0^\infty dr' K_l(r, r')\phi_l(r'),$$

where

$$K_l(r, r') = |V(r)|^{1/2} g_l(r, r')|V(r')|^{1/2}$$

and

$$\phi_l(r) = |V(r)|^{1/2} u_l(r).$$

Thus, the eigenvalues of the Hermitian, positive kernel K_l are the reciprocals of the critical numbers $\lambda_1, \lambda_2, \ldots$ just described.

The trace of K_l is the sum of the eigenvalues,

$$\sum_1^\infty \frac{1}{\lambda_\alpha} = \int_0^\infty dr K_l(r, r) = \frac{1}{2l+1} \int_0^\infty dr\, r|V(r)|.$$

But, if there are n bound states, $\lambda_1 < \lambda_2 \ldots < \lambda_n < 1$, while $0 < \lambda_\alpha \leq \infty$, which supplies the inequality

$$\sum_1^\infty \frac{1}{\lambda_\alpha} \geq \sum_1^n \frac{1}{\lambda_\alpha} > n$$

and the theorem

$$n_l(V) \leq n_l(-|V|) < \frac{1}{2l+1} \int_0^\infty dr\, r\,|V(r)|.$$

When the potential is positive over an appreciable range of r, the upper limit given by this theorem may not be very realistic. Then, one would do better to compare $V(r)$ with a potential that equals $V(r)$ where $V(r) < 0$ but is zero wherever $V(r) > 0$. The same considerations show that

$$n_l(V) < \frac{1}{2l+1} \int dr\, r|V(r)|\,]_{V<0},$$

and now the integration is extended only over the regions of negative $V(r)$. This form of the inequality requires no restriction on the behavior of $V(r)$ in the domain of positive values.

A potential that realizes the upper limit to n_l as closely as one wishes, for any particular l, is given by

$$V(r) = -\sum_{\nu=1}^n V_\nu \delta(r - r_\nu), \qquad V_\nu > 0.$$

The eigenvalue problem is equivalent to the n-dimensional determinantal equation

$$\det[\lambda^{-1}\delta_{\mu\nu} - V_\mu^{1/2} g_l(r_\mu, r_\nu) V_\nu^{1/2}] = 0.$$

and there are just n eigenvalues. We can now choose each of the ratios $r_{\mu+1}/r_\mu$ to be sufficiently large that the nondiagonal elements of the determinant are as small as desired. Furthermore, we make all the diagonal elements equal by requiring of every V_ν that

$$V_\nu g_l(r_\nu, r_\nu) = \frac{1}{2l+1} V_\nu r_\nu \to 1$$

and in this way attain, with arbitrary precision, a single n-fold degenerate eigenvalue at unity. The resulting situation is one with n bound states of essentially zero binding energy and with $\sum \lambda_\alpha^{-1} \cong n$.

The number of bound states is the number of states that lie at or below zero energy, and a similar problem can be posed for any negative energy, $-\kappa^2$. All the previous arguments continue to apply, provided one replaces the Green's function with the one defined by

$$\left(-\frac{d^2}{dr^2} + \kappa^2 + \frac{l(l+1)}{r^2}\right) g_l(r, r', \kappa) = \delta(r - r').$$

The required function is

$$g_l(r, r', \kappa) = \frac{1}{\kappa} S_l(\kappa r_<) E_l(\kappa r_>)$$

where

$$S_l(x) = i^{-l} x j_l(ix)$$

$$E_l(x) = -i^l x h_l(ix)$$

in the notation of spherical Bessel functions. Thus,

$$n_l(E \leq -\kappa^2) < \int_0^\infty dr g_l(r r\kappa)|V(r)| = \int_0^\infty dr \; r|V(r)|(1/\kappa r) S_l(\kappa r) E_l(\kappa r),$$

and

$$\frac{1}{x} S_0(x) E_0(x) = \frac{1}{2x}[1 - e^{-2x}]$$

$$\frac{1}{x} S_1(x) E_1(x) = \frac{1}{2x}\left[1 - \frac{1}{x^2} + e^{-2x}\left(1 + \frac{1}{x}\right)^2\right]$$

for example. Here again, and in the following, integrations only over the regions of negative V can be used.

The function $g_l(rr\kappa)$, and thereby the integral $\int_0^\infty dr g_l(rr\kappa)|V(r)|$, has two characteristics that are physically necessary as properties of the number of states with any specified l below the energy $-\kappa^2$. It is a monotonically decreasing function of κ and of l. On regarding l together with κ as continuous parameters, we deduce from the Green's function differential equation that for any infinitesimal increase of κ or l,

$$\delta g_l(rr\kappa) = -\int_0^\infty dr'[2\kappa\delta\kappa + r^{-2}(2l + 1)\delta l](g_l(rr'\kappa))^2 < 0.$$

We shall apply the monotonic dependence on κ to a situation with n bound states for a given l, so that

$$\int_0^\infty dr g_l(rro)|V(r)| > n \geq 1.$$

Then, there is a unique solution of the equation

$$\int_0^\infty dr g_l(rr\kappa_1)|V(r)| = 1,$$

and

$$n_l(E \leq -\kappa_1^2) < 1,$$

which is to say that the lowest bound state, the ground state for the given l, lies above the energy $-\kappa_1^2$, or

$$E_1 > -\kappa_1^2.$$

The deepest of these ground states is the one for $l = 0$, and a lower limit to the ground state energy is obtained by solving the equation

$$\int_0^\infty dr(1 - e^{-2\kappa_1 r})|V(r)| = 2\kappa_1.$$

For the class of potentials defined by $\int_0^\infty dr|V(r)| < \infty$, we have the crude estimate

$$\kappa_1 < \frac{1}{2}\int_0^\infty dr|V(r)|$$

and $$E_1 > -\frac{1}{4}\left(\int_0^\infty dr|V(r)|\right)^2.$$

The potential

$$V(r) = -V_1\delta(r - r_1), \qquad V_1 r_1 > 1$$

shows that even this limit can be approached as closely as desired, by choosing $V_1 r_1$ to be sufficiently large. In a similar way, the equation

$$\int_0^\infty dr g_l(r r \kappa_m)|V(r)| = m = 2, 3, \ldots \leq n$$

has a unique solution, and

$$n_l(E \leq -\kappa_m^2) < m$$

or $$E_m > -\kappa_m^2.$$

Accordingly, we have obtained lower limits to the energies of all the bound states of a given potential.[3]

The transference of these ideas to an arbitrary nonspin-dependent three-dimensional potential $V(\mathbf{r})$ requires only one major modification. The Hermitian, positive kernel of the integral equation

$$\lambda^{-1}\phi(\mathbf{r}) = \int (d\mathbf{r}')K(\mathbf{r}\mathbf{r}'\kappa)\phi(\mathbf{r}'),$$

is $$K(\mathbf{r}\mathbf{r}'\kappa) = |V(\mathbf{r})|^{1/2}G(\mathbf{r}\mathbf{r}'\kappa)|V(\mathbf{r}')|^{1/2}$$

and the Green's function, which is defined by

$$(-\nabla^2 + \kappa^2)G(\mathbf{r}\mathbf{r}'\kappa) = \delta(\mathbf{r} - \mathbf{r}')$$

and explicitly presented as

$$G(\mathbf{r}\mathbf{r}'\kappa) = \frac{e^{-\kappa|\mathbf{r}-\mathbf{r}'|}}{4\pi|\mathbf{r} - \mathbf{r}'|},$$

implies that the kernel is singular on the diagonal. We therefore shift our attention to the iterated kernel, the trace of which is

$$\int (d\mathbf{r})(d\mathbf{r}')(K(\mathbf{r}\mathbf{r}'\kappa))^2 = \sum \frac{1}{\lambda_\alpha^2},$$

and deduce for a suitable class of potentials that

$$N(E \leq -\kappa^2) < \frac{1}{(4\pi)^2}\int (d\mathbf{r})(d\mathbf{r}')|V(\mathbf{r})|\frac{e^{-2\kappa|\mathbf{r}-\mathbf{r}'|}}{|\mathbf{r} - \mathbf{r}'|^2}V(\mathbf{r}')|,$$

which includes an upper limit to the total number of bound states,

$$N < \frac{1}{(4\pi)^2}\int (d\mathbf{r})(d\mathbf{r}')\frac{|V(\mathbf{r})||V(\mathbf{r}')|}{|\mathbf{r} - \mathbf{r}'|^2}.$$

The latter quantity exists for potentials that decrease more rapidly than $|\mathbf{r}|^{-2}$ as $|\mathbf{r}| \to \infty$ and that in the neighborhoods of a finite number of points $\mathbf{r}_0$ are less singular than $|\mathbf{r} - \mathbf{r}_0|^{-2}$. These statements are to be interpreted by such inequalities as

$$|\mathbf{r}| > R, |V(\mathbf{r})| < C|\mathbf{r}|^{-a}, a > 2.$$

A potential of this class that is spherically symmetrical about its only singularity, at the origin, satisfies the condition $\int_0^\infty dr\, r|V(r)| < \infty$. It should be noted that the upper limit to N, deduced from the individual n_l limits and from the fact that no bound state can occur for $2l + 1 > \int_0^\infty dr\, r|V|$, is

$$N < \frac{1}{2}\int_0^\infty dr\, r|V| \left[\int_0^\infty dr\, r|V| + 1\right].$$

The energy of the ground state is bounded below by

$$E_1 > -\kappa_1^2$$

where κ_1 is the unique solution of the equation

$$\frac{1}{(4\pi)^2}\int (d\mathbf{r})(d\mathbf{r}')|V(\mathbf{r})| \frac{e^{-2\kappa_1|\mathbf{r}-\mathbf{r}'|}}{|\mathbf{r}-\mathbf{r}'|^2} |V(\mathbf{r}')| = 1,$$

given that

$$\frac{1}{(4\pi)^2}\int (d\mathbf{r})(d\mathbf{r}')| \frac{|V(\mathbf{r})||V(\mathbf{r}')|}{|\mathbf{r}-\mathbf{r}'|^2} > 1.$$

More generally, when the last integral exceeds the integer N, the solution of the equation

$$\frac{1}{(4\pi)^2}\int (d\mathbf{r})(d\mathbf{r}')V(\mathbf{r}) \frac{e^{-2\kappa_m|\mathbf{r}-\mathbf{r}'|}}{|\mathbf{r}-\mathbf{r}'|^2} |V(\mathbf{r}')| = m = 1, 2, \ldots N$$

supplies a lower limit to the energy of the m^{th} of the N discrete states,

$$E_m > -\kappa_m^2.$$

There is a simple variant of these procedures that is worth mentioning. A comparison potential to $V(\mathbf{r})$ can be defined that equals $V(\mathbf{r})$ wherever $V(\mathbf{r}) < -\kappa^2$ and equals $-\kappa^2$ in the regions for which $V(\mathbf{r}) \geq -\kappa^2$. The energy values associated with the latter potential are depressed by the amount κ^2 relative to those of the potential that equals $V(\mathbf{r}) + \kappa^2$ wherever this quantity is negative but is zero otherwise. Thus, an upper bound to the total number of states associated with the last potential serves to limit the number of states that lie at or below the energy $-\kappa^2$ for the potential $V(\mathbf{r})$. The explicit statement is

$$N(E \leq \kappa^2) < \frac{1}{(4\pi)^2}\int (d\mathbf{r})(d\mathbf{r}') \frac{|V(\mathbf{r}) + \kappa^2||V(\mathbf{r}') + \kappa^2|}{|\mathbf{r}-\mathbf{r}'|^2}\Bigg]_{V+\kappa^2<0},$$

and, for the states of given l in a spherically symmetrical potential,

$$n_l(E \leq -\kappa^2) < \frac{1}{2l+1}\int dr\, r|V(r) + \kappa^2|\Bigg]_{V+\kappa^2<0},$$

from which we obtain lower limits to the energies of the various states. Note that no matter how slowly the potential approaches zero at great distances, there is a finite upper limit to the number of states that lie at or below any energy $-\kappa^2 < 0$. Of course, as $\kappa \to 0$, this limit will approach infinity if the conditions for a finite total number of states are not satisfied.

As an important example of spin-dependent potentials we consider

$$V(\mathbf{r}) = V_a(r) + V_b(r)S_{12}$$

$$S_{12} = 3\,\frac{\sigma_1\cdot\mathbf{r}\sigma_2\cdot\mathbf{r}}{r^2} - \sigma_1\cdot\sigma_2,$$

which refers to a pair of particles with spin angular momenta $^1/_2\sigma$. In the spin singlet state, $S_{12} = 0$ and we need consider only the triplet state, for which $\sigma_1\cdot\sigma_2 = 1$. We then note the algebraic property $(S_{12} + 1)^2 = 9$, so that the triplet eigenvalues of S_{12} are 2 and -4. Just for simplicity, we shall assume that $V_b(r)$ is everywhere negative, but $V_a(r)$ is not restricted in this way. The notation $A \geq B$ will be used for spin matrices to mean that $A - B$ is a positive matrix, one that can never realize a negative expectation value. Thus,

$$V(\mathbf{r}) \geq V_a(r) + 2\,V_b(r),$$

and the spin-independent spherically symmetrical potential that equals $V_a + 2V_b$ where this quantity is negative and that equals zero otherwise provides a comparison potential to which the preceding three-dimensional considerations can be applied, since no classification of states is involved.

More detailed results can be obtained by considering specific states, such as the even parity, $J = 1$ states, $^3S_1 + {}^3D_1$. The wave function for energy $-\kappa^2$ is described by the pair of radial functions $u_0(r)$, $u_2(r)$ that obey the coupled integral equations

$$u(r) = \int_0^\infty dr' g(rr'\kappa)(-V(r')u(r')).$$

Here,

$$u(r) = \begin{pmatrix} u_0(r) \\ u_2(r) \end{pmatrix} \qquad g = \begin{pmatrix} g_0 & 0 \\ 0 & g_2 \end{pmatrix},$$

and

$$V = \begin{pmatrix} V_a & 2^{3/2}V_b \\ 2^{3/2}V_b & V_a - 2V_b \end{pmatrix}.$$

If we exhibit a matrix, $V_c(r)$, such that

$$V(r) \geq V_c(r)$$

and

$$-V_c(r) \geq 0,$$

the evident matrix generalization of the previous arguments supplies the limit

$$n(E) \leq -\kappa^2 < \int dr\ tr\ [g(rr\kappa) - V_c(r)]$$

where the trace refers to the two-dimensional matrices.

A suitable choice is the comparison potential just described, written as a multiple of the unit matrix, which gives

$$n(E \leq -\kappa^2) < \int_0^\infty dr(g_0(rr\kappa) + g_2(rr\kappa))|V_a(r) + 2V_b(r)|\big|_{V_a + 2V_b < 0}$$

and, in particular,

$$n < \frac{6}{5}\int dr\ r|V_a(r) + 2V_b(r)|\Big]_{V_a + 2V_b < 0}.$$

The latter also implies the alternative bound

$$n(E < -\kappa^2) < \frac{6}{5} \int dr\ r|V_a(r) + 2V_b(r) + \kappa^2| \Big]_{V_a + 2V_b + \kappa^2 < 0}.$$

A somewhat more elaborate treatment follows from the remark that the matrix $V(r)$ defines three regions. In region I, $V_a < -4|V_b|$, and $-V$ is positive definite. Region II is characterized by $2|V_b| > V_a > -4|V_b|$, and here the matrix V is indefinite, while in region III, $V_a > 2|V_b|$ and V is positive definite. For a comparison potential, we use V itself in region I, the multiple $V_a + 2V_b$ of the unit matrix in region II, and zero in region III. There results the upper bound

$$n(E \leq -\kappa^2) < \int_{\mathrm{I}} dr[g_0(r\kappa)|V_a(r)| + g_2(r\kappa)|V_a(r) - 2V_b(r)|] + \int_{\mathrm{II}} dr(g_0(r\kappa) + g_2(r\kappa))|V_a(r) + 2V_b(r)|$$

and

$$n < \int_{\mathrm{I}} dr\ r\left[|V_a(r)| + \frac{1}{5}|V_a(r) - 2V_b(r)|\right] + \frac{6}{5}\int_{\mathrm{II}} dr\ r|V_a(r) + 2V_b(r)|.$$

Again, an alternative limit is obtained for $n(E \leq -\kappa^2)$ on replacing $V_a(r)$ with $V_a(r) + \kappa^2$ in the latter formula, with a corresponding redefinition of regions I and II.

In an application to a physical system, such as the deuteron, for which the distribution of energy values is known, these inequalities provide simple bounds on the potential used to represent the data.

* Supported in part by the Air Force Office of Scientific Research (ARDC).

[1] These PROCEEDINGS, **38**, 961 (1952).

[2] The physical constant $2m/\hbar^2$ is absorbed into the definitions of potential and energy.

[3] Some remarks in a very recent paper, L. Rosenberg and L. Spruch, *Phys. Rev.*, **120**, 474 (1960), footnote 21, indicate that these authors have considered similar questions.

A GLIA-NEURAL THEORY OF BRAIN FUNCTION

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> One day, I suppose, someone will find the clue and we shall then realize that we have been watching the missing mechanism at work in every experiment upon the brain that we did, but never recognized it for what it was.
>
> —B. Delisle Burns[1]

Theories of brain function abound, ranging from Aristotle's idea that it cools the blood to our present notion that its operations make behavior possible. Theories as to what these operations might be are not scarce either, and they extend from Descartes' idea (in which the pineal gland was supposed to move from left to right to permit humors to flow into one or the other of the brain ventricles) to the present-day almost universal view assigning to neurons alone the critical role. This neuron theory has generated much valuable information about brain function during the

past 75 years. The essay to follow suggests that a brain-model in which we visualize the glia and the neurons working together as the fundamental functional unit may provide an even more useful scheme for students of brain function to carry with them into their experiments.

Comparative anatomy demonstrates with reasonable clarity that every "brain," whether invertebrate or vertebrate, contains cells of two types—neurons and non-neurons. These latter we may for convenience call glia cells, bravely risking thereby the wrath of anatomists who would not include them all under that heading and disregarding the fact that many varieties of glia exist. A brain like our own contains vast systems of such glia cells, and they outnumber the neural elements perhaps tenfold. In electron microscope pictures, the area covered by these ubiquitous cells may exceed by a large factor that occupied by nerve cells and their processes. Hence glial cell cytoplasm takes up an astonishingly large fraction of brain volume, a fact never fully appreciated in the era of light microscopy with its cells badly shrunken by fixatives. An untutored observer constructing for himself a model of the brain from electron microscope pictures alone might well describe it as a huge collection of glia cells through which a nerve process occasionally wanders; those of us well instructed in the conventions, however, see a marvelously intricate arrangement of nerve cells held together by relatively unimportant non-neural elements. The point of view this essay urges is some golden mean between these two extremes, for this will be required if the reader is to evaluate the idea that glia cells might play an important role in the mechanisms responsible for learned and unlearned behavior. So much, then, for the anatomical facts upon which any useful model of brain function must solidly stand.

Naturalists, psychologists, physicians, and the man in the street know of many aspects of animal and human behavior for which a neural basis cannot now be provided. Let us consider merely one of these—hibernation in animals—as an example of the entire class. The bat, hamster, ground squirrel, and several other mammals periodically enter a cool place, drop body temperature, and go into what appears to be a deep sleep. In the laboratory, furthermore, the body temperature can be dropped well toward zero degrees centigrade, at which point neural activity in the brain also approaches zero and yet several complex adaptive responses persist.[2] When the animal wakens again, he proceeds as if nothing had happened to his nervous system at all, promptly exhibiting his normal repertoire of complex behavior, much of which must have been previously learned. Near-abolition of nervous activity thus fails at every point to destroy his most precious possession, knowledge of what to do to survive and to reproduce.

For many a professional neurophysiologist, a highly embarrassing fact of life is his failure thus far to deduce from the known properties of neurons any satisfactory conception of how this storage and retrieval of memories takes place. He has industriously assembled an impressive body of data pertaining to nerve excitation, conduction, and synaptic transmission, yet none of it relates very convincingly to such an obvious event as instant recall of a face once seen. Worse yet, the enormous repertoire of behavioral responses with which his new-born baby comes equipped—breathing, swallowing, crying, and sleeping, for instance—completely eludes his every effort.

The neurophysiological theorist today is wedded to the idea that neurons alone

regulate the responses that organisms emit. Yet, from what we know of neurons, they operate on a time base of milliseconds or seconds at the most, and the behavioral patterns handed down from one generation to the next or acquired as a habit and retained for a lifetime require some stable, utterly dependable storage system operating on a time base of hours, years, and generations. Millisecond neurophysiology, however arranged and rearranged thus far by its supporters, cannot clearly describe the mechanism of this storage to anyone's complete satisfaction. It is a curious fact that the glia cells, which could conceivably serve as that stable storage system, have apparently never seriously been assigned the chore. If, in any event, someone has developed the suggestion Nansen advanced in 1886[3] when he said neuroglia was "the seat of intelligence, as it increases in size from the lower to the higher forms of animal," his message has thus far had no impact whatever upon the main stream of western—or eastern—research on the brain.

In order to provoke some experiments, then, let me put Nansen's thought in strong modern words. The glia cells act in some unknown manner to organize neurons. They provide the basis for the "fields," "cell assemblies," and similar conceptions so many biologists—experimental and "theoretical"—have been forced to postulate. The electron microscope shows glia to invest, surround, and attach itself to nerve soma, axons, and dendrites—out to the finest terminals in the neuropil of C. Judson Herrick—and this may be so because that arrangement is precisely what enables neurons to transmit coherent, organized messages. Glia could receive afferent impulses, organize them somehow before permitting efferent outflow, and in still other ways yet to be discovered intervene so as to give order to neural events. A brain without glia would, in this conception, be a giant computer operating at random for lack of a program.

This notion is not to be confused with the far less comprehensive ideas expressed by Cajal and others who conceive the glia as a kind of insulator, especially at synapses. Nor do I mean that glia merely physically supports or metabolically nourishes neurons as has so often been suggested.[4] Perhaps glia does all these things, but in addition it would somehow "tell" the neuronal masses what they are supposed to do—in the same sense, I suppose, that the computer program "tells" its digital units what order and sequence of processes they must execute.

How, one may ask, could the glia cells become so wise? An answer buttressed by experiment does not come easily, but then neither does one that tells us how the undifferentiated cell develops during embryology into a liver or a gonad. Genetic mechanisms residing in glia cells could cause them to organize my neurons so that I breathe and remember just as genetic mechanisms actually keep my hair growing out brown in color year after year. This statement is not confirmed—nor is it contradicted—by any experimental fact I know.

When an embryo develops its first neurons, it also develops its first glia cells, and one can imagine that a mutual interaction between them, and a division of the labor they will always share, starts at that point in time. The remarkable findings of Weiss, Sperry, and others on regeneration of function in sense organs and motor apparatus[5] seem pertinent here. Their facts are simple: surgical operations on amphibia and fish can make chaos out of the normal neural connections (in brain and spinal cord) yet somehow fail to prevent normal function. For years, neither I nor anyone else has been able to understand how an eye, removed from its socket

and transferred to the opposite side, comes to act after its nerve regenerates as if it were still in its original site. But what if I suppose the glia of the retina or optic tectum "knows" where its neurons are supposed to be and how they are to act? The glia would then physically guide and physiologically control the neurons in that eye from beginning to end of the animal's life, and the surgical intervention of the experimenters merely provides another opportunity for that glia to do what its genes demand. When investigators report experiments proving Schwann (glia) cells "innervate" frog muscle—and give clear evidence of making functional connections with it—shortly after the peripheral nerve has been cut,[6] I can see an interesting clue to the way glia prosecutes its business. And when other investigators conclude from microelectrode studies that the frog's eye "speaks to the brain in a language already highly organized and interpreted instead, of transmitting some more or less accurate copy of the distribution of light on the receptors,"[7] this too becomes comprehensible, because according to the idea present here, glia would somehow organize the moment-by-moment activity of neurons as well as shepherd its flock of them mechanically and otherwise in embryology and regeneration. The experiments show regenerating optic fibers unquestionably do know exactly where to go and what to do in the brain and the authors insist that there is a "genetically determined representation of the world built into the tectum."[8] If I suppose the whole mysterious orderliness reduces to an expression of genetic properties of glia cells, there can be as much harmony and beauty here as when I suppose the neurons do it by themselves.

There is a host of other data, all well known, that strikes me as worth re-examining within this new conceptual framework. Many of us who record brain waves and the like find great difficulty getting the slow-wave (including DC) activity of the brain to emerge clearly out of mere nerve membrane-potential changes. In 1951, for instance, my colleagues and I were puzzled upon close examination of microelectrode records that an evoked slow wave could not possibly be a summation of nerve-cell electrical activity.[9] We did not then know what is now so clear from the electron microscope pictures—that a microelectrode tip must lie either inside neurons or inside glia cells and that there is no such thing as "extracellular recording" from brain and spinal cord. Suppose we hold the glia cells responsible for the slow waves and neurons for the spikes; here is a generalization that almost certainly cannot be exactly correct, but having boldly made it, we can now devise appropriate experiments to test it.

The latest fashion in brain research brings the electrical recording of brain waves to the problems of learning. An outstanding result is the discovery in the midbrain reticular formation of rhythmical activity that preserves the temporal features of the stimulus.[10] For instance, if you teach a cat that a light flashing seven times per sec means move or get shocked, you are likely to find a 7-per-sec brain wave present even in the dark. What brain structures give rise to this durable new brain wave? Conventional neurophysiological doctrine dictates that potential variation in many neuronal membranes is responsible, but I see no reason why an alternate theory in which a glia-neural complex of cells forms the functional unit cannot also be considered.

Now let us look at the cortical brain waves with which Berger's name is attached. They resemble in every respect—frequency spectrum, amplitude, variability, etc.—

waves recordable in gray and white matter anywhere else in the brain, including the new ones generated in midbrain during learning. A good many purely neuronal theories exist to deal with the cortical brain wave activity, many recent ones leaning heavily upon partial depolarizations in vertically oriented terminal dendrites. The only direct evidence that glia could help give the brain wave comes from Tasaki and Chang,[11] who showed that astrocytes produce an electrical wave, but the idea that glia might be involved, though suggested several times, seems not to have got very far.[12] Is it, however, a completely unreasonable thought? The neurons in all these areas from which brain waves are recorded vary enormously in size, distribution, number, and arrangement. How can the same electrical spectrum and amplitude possibly arise from axons in white matter and from nerve cells so diverse in the way synaptic buttons end upon them, in the detailed arrangement of their dendrites and axon terminals, and even in such physiological properties as complete or incomplete depolarizability? Perhaps that electrical activity displayed in common by such regions arises not so much from neurons as from those *other* cells that they share in common, the glia.

There is a phenomenon called spreading cortical depression with which neurophysiological theory has struggled for many years. In its presence, among other things, the animal cannot perform learned responses, the EEG is abolished, and a DC potential normally present between the ventricle and the cortical surface disappears. No comprehensive treatment of this constellation of facts has ever arisen from the neuron theory alone. If, however, we assume glia creates steady potentials and also displays electrical waves that signal its processes of organizing neurons, spreading depression, along with several other cortical electrical abnormalities, could conceivably be fitted into a realistic theoretical framework. Those who deal with human brain waves associated with sleep, brain tumors, epilepsy, and the like may find that this idea will illuminate their problems and lead to some experiments of value in their work.

Let me continue this recital with two experiments so new they have not yet even been published. These are the straws that broke the camel's back, so to speak, and led to my writing this paper. This first was reported by Dr. Frank Morrell on October 20, 1960. With a microelectrode in the rabbit visual cortex he isolated a unit that discharged with a single burst of impulses to a flash of light. He then passed a weak current through the cortex while stimulating the eye with flashes at 5 per sec. Several minutes later, and with the current still flowing, he retested the animal with a single flash of light. The unit now responded not with a single burst but with a train of them at 5 per sec. As time passed, its tendency to give this complicated response to a single flash diminished, and after a half hour the single flash elicited the expected single burst. He thus elegantly demonstrated what actually had previously been shown,[13] namely, that a weak direct current reversibly modifies neuronal activity for a considerable period if the conditions are right. I am incapable of imagining how this phenomenon, which appears so closely related to learning, can be explained simply by supposing modifications of neuronal membranes. It is somehow much easier for me to imagine temporary alteration in the properties of the glia cells near to his electrodes. Either view, of course, is an entirely unsubstantiated guess, and an experimenter may take one or the other of them as the basis for his further investigations of the phenomenon.

On October 26, Dr. Gunnar Svaetichin reported the second experiment, a carefully controlled study of the electrical responses of a fish retina. He presented convincing evidence that one of three types of electrical response elicited by illumination appears only when the microelectrode lies inside a large horizontal cell, which is a glial cell in this retina. Another appeared only after the electrode had penetrated a Muller fiber, which Cajal himself identified as a glia cell. Both these forms of activity are sustained potential shifts, not spikes. Typical spikes can be recorded, of course, from the ganglion cells and optic nerve fibers.

It may be instructive now to consider how the glia might be involved in electroshock and in other brain stimulation studies, limiting consideration here to the so-called self-stimulation experiments that prove animals will go to a great deal of trouble in order to receive brain shocks.[14] That a monkey should press a key every few seconds for days, barely pausing for food and sleep, when the only result of this is to cause electrical shocks to be delivered deep inside his brain is certainly an observation to challenge any neurophysiological theory. Close examination shows neither the shock parameters (frequency, duration, and wave form) nor the exact brain area (within broad limits) to be highly critical factors. Shock intensity is, however, important, being linearly related over a reasonable range to the strength of the response according to recent as-yet unpublished data of Eliot S. Valenstein.

The prevailing view on how these shocks produce the brain events responsible for the compelling, complex, precise behavior invokes nerve-membrane depolarizations in the great limbic-midbrain neuronal system that recent research implicates in emotions, motivation, and the like. In short, the shocks stimulate nerve fibers and nerve cells. Many awkward assumptions are required, however, to deal with such matters as current spread, the loose requirements of stimulus parameters, the way neurons interact at synapses, etc. Let us however imagine the substrate upon which the shocks act to be primarily the great sheet of glia cells surrounding the soma and fiber tracts of the limbic-midbrain circuit. The shocks now would first activate this glia system (which incidentally would turn out to be much less finicky than neurons as far as shock parameters go), and the glia in turn would selectively and appropriately activate its neural compliment. The output of this joint effort looks like normal behavior because the shocks set off the same glia processes that occur in normal behavior. This construction placed upon the facts seems no more remote and unreasonable as a working basis than a hypothesis based upon the neuron doctrine alone.

What Karl Lashley might say of the idea that his engrams reduce to the genetic properties of glia cells would be interesting to know. He died baffled by the knowledge that no sensible relation exists between the location and extent of brain lesions on the one hand and the amount and kind of behavioral deficit these produce on the other. Superficial knife cuts that must utterly demolish intracortical neuronal organization make surprisingly little difference to behavior. Would he agree to the guess that this is so because the cortical glia cannot easily be damaged with a knife and that it restores itself after such a lesion, begins rearranging neurons, and does its best with whatever it can find? One gathers Heinrich Klüver might be favorably disposed to such a view, for in discussing the text of Lashley's final communication, he says:

> ". . . it is of interest to recall that a famous physician Schleich, about 30 years ago wrote a book, *Vom Schaltwerk der Gedanken*, in which he tried to explain all psychological activities on the basis of neuroglial activities and in which he related thinking, remembering, forgetting, imagination, action, etc., to the contraction or relaxation of neuroglial processes. You may wonder whether Schleich's glial switchboard is really such a fantastic idea after you have seen Pomerat's tissue cultures containing cells with rhythmical pulsatile activity—cells which have been identified as oligodendroglial cells. The question may be raised whether psychoneurology—which at the present time seems to be chiefly 'psychoneuronology'—can afford to ignore the possibilities of a 'psychogliology,' so much the more since man, according to Friede, may be defined as the animal with the highest glial index."[15]

One additional thought needs development in conclusion. Desmedt and La-Grutta[16] report experiments showing that inhibitors of pseudocholinesterase, an enzyme localized in the glia, profoundly influence behavior, brain waves, and evoked response amplitude in the cat. This may be the first study clearly showing participation of glia in brain function, and its implications did not escape the investigators although they seem not to have expressed the central idea of this paper.

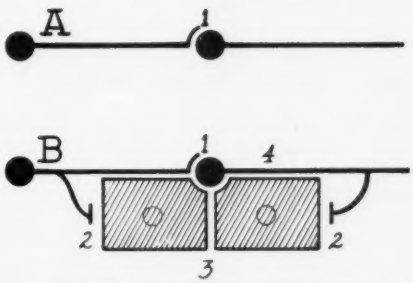

FIG. 1.—Diagrams showing elementary units of neural function according to present generally-accepted conventions (*A*) and the idea formulated here (*B*), which adds to synapses (1) what might be called gliapses, the neurono-glial (2), glia-glial (3), and glia-neural (4). All anatomical contacts between neurons and glia cells portrayed in *B* appear abundantly in electron microscope pictures of brain and spinal cord.

If this idea is correct in principle, glia must show exquisite sensitivity to a wide range of chemical substances and it doubtlessly manufactures them itself. One wonders therefore to what degree it is glia cells, not neurons, that react to excess hydrogen ion in the medullary respiratory center, drop out of action with barbiturate anesthetics, produce the depression of spinal reflexes in alcohol intoxication, and respond promptly to high blood levels of a sex hormone. Such chemicals do not influence all brain regions equally, and since there are many pharmacologically active substances, one must postulate in this scheme many classes of brain glia differentiated from one another on the basis of their biochemical specificities.

A brain map identifying glia-collections in terms of these biochemical specificities would be an intriguing new way to visualize and thus classify brain structures. Such a glia-anatomy would present the brain in the form of balls, sheets, rods, and complex 3-dimensional surfaces packed tightly together into a volume having the final external outline of the brain itself. To what extent the solid volumes outlined in such a glia-anatomy would coincide with the brain subdivisions conventionally taught in neuroanatomy is an interesting question. One might hope the coincidence would be exact, for otherwise medical students who already have such difficulty learning the way one great class of brain cells—neurons—are put together might rebel at having to learn an entirely different anatomical plan for the other—glia.

To summarize: This paper outlines a view of the brain diagrammed in Figure

1 in which its two cellular components—the neurons and the glia—mutually collaborate to produce behavior. Glia is here conceived as genetically charged to organize and program neuron activity so that the best interests of the organism will be served; the essential product of glia action is visualized to be what we call innate and acquired behavioral responses. In this scheme, neurons in large part merely execute the instructions glia give them.

This view arises as an inference from some old and modern data of anatomy, physiology, and the behavioral sciences. That powerful new anatomical tool, the electron microscope, shows glia to comprise a very large part of brain tissue; since no one knows what this glia mass does, one is at liberty to assign to it, conceptually, an essentially passive role, as is conventional, or a most highly active one, as is suggested here. Neurophysiology, dominated by the neuron theory of Cajal, has generated over the past 50 years a mountain of data without being able to formulate a convincing explanation for even such a commonplace behavioral event as remembering a name. Its data, furthermore, repeatedly imply that something else besides mere neuronal activity is at work. Could the "something else" needed to pull together disparate facts, harmonize apparent contradictions, and put an end to our journeys down blind alleys just be the physiological properties of that other cell population of the brain, the glia?

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add'l 100's	2.20	4.40	6.60	3.05

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